

REMARKS

The Office Action dated January 17, 2002 has been received and reviewed. Claims 1 through 20 are pending in the present application. Claims 5 and 13 have been withdrawn from consideration. Claims 1 through 4, 6 through 12 and 14 through 20 stand rejected. The application is to be amended as previously set forth. All amendments are made without prejudice or disclaimer. Reconsideration is respectfully requested.

1. **Priority**

Applicants claim priority from an International Patent Application PCT/EP99/07800 filed 06 October 1999 designating the United States of America, which itself claims priority from European Patent Application EP 98203529.7 filed 20 October 1998. Applicants hereby acknowledge the Examiner's remarks regarding receipt of the required foreign priority document and acknowledge that unless such document is filed as provided in and required by 35 U.S.C. § 119 (a)-(d) and 37 C.F.R. § 1.55, enjoyment of the benefit of foreign priority to the EPO document will be denied. A copy of the priority document has been requested and will be supplied upon receipt by the undersigned.

2. **Election/Restrictions**

Applicants hereby affirm the election without traverse made during a telephone conversation between the Examiner and Mr. Allen Turner on December 12, 2001 to prosecute the invention of Group I, claims 1-20 and the species election encompassing *Lactococcus* sp, Crohn's disease, immunosuppressive drugs and IL-10. The pending claims have been amended herein to reflect this election. Applicants further acknowledge that claims 5 and 13, as well as the non-elected species, have been withdrawn from further consideration by the Examiner according to 37 C.F.R. § 1.142(b).

3. **Claim Objections**

Claims 9 and 17 have been objected to for reciting a grammatically improper phrase. Specifically, the Examiner has objected to the phrase "co-administration of the at least . . .". Claims 9 and 17 have been amended as suggested by the Examiner to instead recite the phrase "co-administration of at least . . .". Applicants respectfully submit that the objection to claims 9 and 17 has been obviated and, accordingly, request withdrawal thereof.

4. **Claim Rejections under 35 U.S.C. § 112, first paragraph**

Claims 1 through 4, 6 through 12 and 14 through 20 have been rejected under 35 U.S.C. § 112, first paragraph. Specifically, the Examiner has stated that while the specification is enabled for 1) a method of treating colitis in a mammal, comprising administering a medicament comprising an amount of a genetically modified *Lactococcus lactis* expressing IL-10 to a mammal with colitis by injecting the recombinant *Lactococcus lactis* into the peritoneum of the mammal, wherein the administration results in an increase in colon length and reduced epithelial damage and infiltration of lymphocytes in the mammal with colitis; and 2) a method as recited in claim 1, wherein the medicament is administered in combination with at least one additional anti-colitis drug, the specification assertedly is not enabled "for other claimed embodiments embraced by the breadth of the claims." (Office Action, pages 8 and 9) Applicants respectfully traverse the rejection for the following reasons.

First, applicants respectfully traverse the Examiner's assertion that the present invention is "directed to a method of treating a mammal by methods of ex vivo gene therapy . . .". (Office Action, page 9) *Ex vivo* gene therapy involves extracting cells from the subject to be treated, genetically altering the extracted cells and transplanting the genetically altered cells back into the subject. See, *Nature*, Vol. 389, pp. 239-242, 1997 (hereinafter the "Verma reference") at p. 240. The present invention does not relate to such *ex vivo* gene therapy, but rather relates to recombinant bacteria introduced into the gastrointestinal tract of the subject to be treated which produce, *in situ*, a cytokine which aids in the prevention or healing of inflammatory bowel disease. Accordingly, it

respectfully is submitted that the cited references relating to the state of the art regarding vectors and methods useful for gene therapy are not relevant to the present invention. Specifically, it is submitted that the Anderson reference (*i.e.*, *Nature*, Vol. 392, pp. 25-30, April 1998) and the Verma reference are not relevant to that which is recited in the claims herein.

Further, applicants respectfully submit that the present invention is not related to DNA vaccination. Accordingly, the state of the art regarding DNA vaccinations which is taught by the McCluskie reference (*i.e.*, *Molecular Medicine*, 5, pp. 287-300, 1999) also is not relevant to the claims of the present application.

As claimed, the present invention relates to the use of a medicament comprising an amount of a cytokine-producing non-invasive Gram-positive bacterial strain for treating inflammatory bowel disease. Particular Gram-positive bacterial strains for which the present invention may be used include *Bacillus subtilis*, *Streptococcus gordonii*, *Staphylococcus xylosum* and *Lactobacillus species* such as *L. bulgaricus*, *L. salivarius*, *L. casei*, *L. helveticus*, *L. delbrueckii* and *L. plantarum*. (See, specification, para. [0038].) All of the bacterial strains listed above are non-invasive strains. The present invention is not intended to suggest using harmful bacterial strands such as *Staphylococcus aureus*, *Enterococcus spp* and *Streptococcus pneumoniae* and the Examiner's point in this regard is well-taken. (See, Office Action, p. 12) Independent claim 1 and dependent claims 3 and 11 have been amended herein to clarify that only non-invasive Gram-positive bacterial strands are intended to be encompassed by the present invention. Applicants respectfully submit that the state of the art does provide sufficient guidance for one skilled in the art to make and/or use a representative number of non-invasive Gram-positive bacteria, such as those listed above, in the method of the present invention.

The surprising results obtained by the present invention relate to introducing any molecule known to affect inflammatory bowel disease into the gut of the subject to be treated so that the molecule can be locally produced by a bacterium that acts as a carrier and as a production unit. Thus, the present invention teaches a method for introducing *any cytokine* into the gut. Applicants respectfully submit that one skilled in the art would be able, without undue experimentation, to

extrapolate from the *L. lactis* examples provided by the present invention to other non-invasive Gram-positive bacteria in the method of the present invention. The specification provides ample guidance to a person skilled in the art regarding how to treat a subject with inflammatory bowel disease by administering a suitable Gram-positive bacterium (*e.g.*, a non-invasive Gram-positive bacterium) as a carrier/production item for any cytokine known to affect the prevention or cure of inflammatory bowel disease.

Further, it respectfully is submitted that the mouse model disclosed by the present invention is a generally recognized model for treatment of inflammatory bowel disease in any subject. In support of this contention, an article authored by the inventors of the present invention and published in the renowned journal *Science* (*Science* 2000: 1352-1355) submitted herewith as Appendix D. It is stated in the abstract of the article that "[t]his approach may lead to better methods for cost-effective and long-term management of IBD *in humans*" (emphasis added). A statement such as this undoubtedly would not be published in a renowned peer-reviewed journal like *Science* if the extrapolation from mice to other mammals was unacceptable to those of skill in the art.

Applicants respectfully submit that the culmination of the above remarks establishes that a person skilled in the art is given sufficient guidance in the present application regarding how to treat any subject with inflammatory bowel disease by administering a suitable non-invasive Gram-positive bacterium as a carrier/production item for any cytokine known to affect prevention or cure of the disease. Accordingly, reconsideration and withdrawal of the 35 U.S.C. § 112, first paragraph, rejection of claims 1 through 4, 6 through 12 and 14 through 18 thus respectfully is requested. Claims 19 and 20 have been cancelled by way of the present amendment and thus the rejection as to these claims has been rendered moot.

5. **Claim rejections under 35 U.S.C. §102**

Claims 1 through 4, 6, 10 through 12, 14 and 18 through 20 have been rejected under 35 U.S.C. § 102(b) as being anticipated by WO 96/11277 to Tagliabue et al. (hereinafter the "Tagliabue

reference") as evidenced by Herfarth et al. (hereinafter the "Herfarth reference"). Applicants respectfully traverse the rejection.

As amended herein, independent claim 1 recites a method of treating inflammatory bowel disease in a subject, the method comprising administering to the subject a medicament comprising an amount of a cytokine-producing non-invasive Gram-positive bacterial strain, *wherein administering the medicament results in reduction of inflammation by at least 50% and/or prevention of onset of inflammation*. The emphasis indicates a limitation added by virtue of the present amendment, support for which may be found in paragraph [0041] of the as-filed specification.

Applicants respectfully submit that the Tagliabue reference neither expressly nor inherently suggests a method of treating inflammatory bowel disease having each of the elements recited in independent claim 1. Specifically, the Tagliabue reference does not suggest, either expressly or inherently, that administration of a cytokine-producing non-invasive Gram-positive bacterial strain results in a reduction of inflammation of at least 50% during inflammatory bowel disease and/or in prevention of the onset of inflammatory bowel disease as is specifically recited by the present invention.

The present invention solves the problem of administering an engineered bacterium which can efficiently prevent and/or cure (by at least 50%) inflammation during inflammatory bowel disease. Faced with this problem, one of ordinary skill in the art would have to undergo an undue amount of experimentation in light of the Tagliabue reference to solve the problem. Moreover, one skilled in the art certainly could not predict that inflammatory bowel disease could be efficiently prevented or cured at all consulting only the Tagliabue reference and the state of the art generally prior to the present invention.

The Tagliabue reference recognizes the problem of surpassing a gastric environment in case bacteria are administered orally and states that spore-forming bacteria *should* reach the intestinal mucosa (*see*, p. 12, lines 13-23, emphasis added). However, the Tagliabue reference fails to offer any examples demonstrating how (and how many) bacteria should be orally administered, if any

bacteria will pass the gastric environment, or if bacteria which do pass the stomach will still be able to produce *de novo* a therapeutically effective amount of protein. Furthermore, the Tagliabue reference shows that administration of IL-1ra-producing *Bacillus subtilis* bacteria directly into the colon via a catheter reduces mortality due to endotoxic shock by 30% to 40% depending upon the dose of LPS (see, pp. 41-42, example 6 and table 4). However, the Tagliabue reference does not disclose any studies concerning oral administration, how to treat inflammatory bowel disease, or what to expect if inflammatory bowel disease were to be treated. The Tagliabue reference merely mentions (p. 17, lines 22-31) that inflammatory bowel disease is a type of disease which is treatable, among eleven other types of diseases, by administration of the pharmaceutical compositions described in that reference. Applicant respectfully submits that this alone does not permit one skilled in the art to draw the conclusion that the onset of inflammatory bowel disease can be prevented or that inflammation due to the disease can be reduced by at least 50%.

Thus, a person of ordinary skill in the art could certainly not predict if treatment with engineered bacteria would, with a reasonable expectation of success, efficiently prevent or cure (by at least 50%) inflammatory bowel disease. Thus, based upon the undue amount of experimentation that would be required based only upon the Tagliabue reference and the state of the art generally prior to the present invention, and there being no reasonable expectation of success, it is clear that the present invention is novel over the prior art of record. Applicants further submit that the teachings regarding exogenous IL-10 as applied to colitis assertedly disclosed in the Herfarth reference do not, when combined with the Tagliabue reference, result in the latter reference anticipating the present invention for the reasons discussed above.

Independent claim 1 is believed to be in condition for allowance and applicants, accordingly, respectfully request withdrawal of the 35 U.S.C. § 102(b) rejection thereof. Each of claims 2 through 4, 6, 10 through 12, 14 and 18 depend, either directly or indirectly, from independent claim 1 and thus are believed to be in condition for allowance for at least the above-stated reasons. As such, applicants respectfully request withdrawal of the 35 U.S.C. § 102(b) rejection of these claims

as well. Claims 19 and 20 have been cancelled herein and thus the rejection of these claims has been rendered moot. Favorable action respectfully is requested.

6. **Claim rejections under 35 U.S.C. §103**

Claims 1, 2, 7, 8, 9, 15, 16 and 17 have been rejected under 35 U.S.C. § 103(a) as being unpatentable over the Tagliabue reference taken in combination with either *The Gastroenterologist*, Vol. 3, 1995, pp. 141-152 (hereinafter the "Korelitz reference") or U.S. Patent 6,262,119 to Ferrante et al. (hereinafter the "Ferrante reference"). As a *prima facie* case of obviousness under 35 U.S.C. § 103 cannot be established based upon the Tagliabue, Korelitz or Ferrante references, or any combination thereof, applicants respectfully traverse the rejection.

In order to establish a *prima facie* case of obviousness under 35 U.S.C. § 103, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference(s) or to combine the reference teachings. "The mere fact that reference can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination [or modification]." *In re Mills*, 916 F.2d 680, 1 USPQ2d 1430 (Fed. Cir. 1990). *See also, ACS Hospital Systems, Inc. v. Monteffiore Hospital*, 732 F.2d 1572, 1577, 221 USPQ 929, 933 (Fed. Cir. 1984) (stating that "[o]bviousness cannot be established by combining the teachings of the prior art to produce the claimed invention, absent some teaching, suggestion or incentive supporting the combination."). Second, there must be a reasonable expectation of success. Third, the cited prior art reference must teach or suggest all of the claim limitations. Furthermore, the suggestion to make the claimed combination and the reasonable expectation of success both must be found in the prior art and must not be based upon applicants' disclosure. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

In light of the foregoing, applicants respectfully submit that the Examiner has failed to establish a *prima facie* case of the obviousness of the present invention based upon the Tagliabue reference, the Korelitz reference, the Ferrante reference, or any combination thereof.

As previously stated, amended claim 1 of the present invention recites a method of treating inflammatory bowel disease in a subject, the method comprising administering a medicament comprising an amount of a cytokine-producing non-invasive Gram-positive bacterial strain to the subject, wherein administering the medicament results in reduction of inflammation by at least 50% and/or prevention of onset of inflammation.

First, the cited prior art references fail to teach or suggest all of the limitations of independent claim 1. As discussed previously, the Tagliabue reference neither teaches nor suggests a method of treating inflammatory bowel disease wherein administering the medicament results in reduction of inflammation by at least 50% and prevention of onset of the inflammation. Applicants respectfully submit that the Korelitz reference and the Ferrante reference similarly lack such teaching or suggestion. Furthermore, none of the references is relied upon for such teaching by the Examiner.

Further, no suggestion or motivation exists, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the references or to combine the reference teachings to achieve a method of treating inflammatory bowel disease wherein administering the medicament results in reduction of inflammation by at least 50% and prevention of onset of inflammation. The mere fact that the references can be combined and/or modified does not render the resultant combination obvious absent some suggestion concerning the desirability of the combination and/or modification. Further, applicants respectfully submit that, even if the cited references were combined, an undue amount of experimentation would be necessary to achieve a method as recited in the present invention and that one skilled in the art would not reasonably expect success upon such modification or combination. (*See supra*)

In view of the foregoing, it respectfully is submitted that a *prima facie* case of obviousness of independent claim 1, as amended herein, cannot be supported based upon the cited references. Accordingly, claim 1 is believed to be in condition for allowance and applicants respectfully request withdrawal of the 35 U.S.C. § 103(a) rejection of this claim. Each of claims 2, 7 through 9 and 15 through 17 depend, either directly or indirectly from independent claim 1 and thus are believed to



be in condition for allowance for at least the above-cited reasons. Favorable action respectfully is requested.

CONCLUSION

In view of the present amendment and the above remarks, claims 1 through 4, 6 through 12 and 14 through 18 are believed to be in condition for allowance and an early notice thereof respectfully is solicited. Should the Examiner determine that additional issues remain which might be resolved by a telephone conference, he respectfully is invited to contact applicants' attorney at the address or telephone number given herein.

Respectfully submitted,

A handwritten signature in black ink, appearing to read "Allen C. Turner", with a long horizontal flourish extending to the right.

Allen C. Turner  
Registration No. 33,041  
Attorney for Applicants  
TRASK BRITT, PC  
P. O. Box 2550  
Salt Lake City, Utah 84110-2550

Date: April 17, 2002

Enclosures:      Appendix A  
                     Appendix B  
                     Appendix C  
                     Appendix D

# **APPENDIX A**

**(MARKED-UP VERSION OF CLAIMS TO SHOW CHANGES MADE)**  
**(Serial No. 09/838,718)**

### Version of Claims with Markings to Show Changes Made

1. (Amended) A method of treating inflammatory bowel disease in a subject, said method comprising:

administering a medicament comprising an amount of a cytokine-producing non-invasive Gram-positive bacterial strain ~~or a cytokine antagonist-producing non-invasive Gram-positive bacterial strain~~ to said subject, wherein the administration of said medicament results in one or more of reduction of inflammation by at least 50% and prevention of onset of said inflammation.

2. (Amended) The method according to claim 1 wherein the cytokine ~~or cytokine antagonist~~ is selected from the group consisting of IL-10, a soluble TNF receptor or another TNF antagonist, an IL-12 antagonist, an Interferon- $\gamma$  antagonist, an IL-1 antagonist, a virus-coded cytokine analogue, and EBV BCRF1.

3. (Amended) The method according to claim 1 wherein the non-invasive Gram-positive bacterial strain is a *Lactococcus* species.

6. (Amended) The method according to claim 1 wherein the bowel disease is selected from the group consisting of chronic colitis, Crohn's disease and ulcerative colitis.

8. (Amended) The method according to claim 7 wherein the at least one therapeutic agent is selected from the group consisting of corticosteroids, sulphasalazine, derivatives of sulphasalazine, includes at least one immunosuppressive drugs, cyclosporin A, mercaptopurine,

~~azathioprine, and another cytokine drug.~~

9. (Amended) The method according to claim 7 wherein the co-administration of the at least one additional therapeutic agent is sequential or simultaneous.

10. (Amended) The method according to claim 1 wherein the medicament is delivered through *in situ* synthesis by recombinant ~~*L. Lactococcus*~~ *lactis*.

11. (Amended) The method according to claim 2 wherein the non-invasive Gram-positive bacterial strain is a *Lactococcus* species.

14. (Amended) The method according to claim 2 wherein the bowel disease is ~~selected from the group consisting of chronic colitis, Crohn's disease and ulcerative colitis.~~

16. (Amended) The method according to claim 15 wherein the at least one therapeutic agent ~~is selected from the group consisting of corticosteroids, sulphasalazine, derivatives of sulphasalazine, includes at least one immunosuppressive drugs, cyclosporin A, mercaptopurine, azathioprine, and another cytokine drug.~~

17. (Amended) The method according to claim 15 wherein the co-administration of the at least one additional therapeutic agent is sequential or simultaneous.

18. (Amended) The method according to claim 2 wherein the medicament is delivered through *in situ* synthesis by recombinant ~~*L. Lactococcus*~~ *lactis*.

19. (Cancelled)

20. (Cancelled)

# **APPENDIX C**

**(VERSION OF SUBSTITUTE SPECIFICATION EXCLUDING CLAIMS  
WITH MARKINGS TO SHOW CHANGES MADE)  
(Serial No. 09/838,718)**

PATENT  
Attorney Docket 4779

NOTICE OF EXPRESS MAILING

Express Mail Mailing Label Number: EL740535351US

Date of Deposit with USPS: April 19, 2001

Person making Deposit: Daniel Thatcher

APPLICATION FOR LETTERS PATENT

for

**USE OF A CYTOKINE-PRODUCING *LACTOCOCCUS* STRAIN TO TREAT  
COLITIS**

Inventors:

Lothar Steidler  
Erik R. Remaut  
Walter Fiers

Attorney:  
Allen C. Turner  
Registration No. 33,041  
Jarett K. Abramson  
Registration No. 47,376  
TRASKBRITT, PC  
P.O. Box 2550  
Salt Lake City, Utah 84110  
(801) 532-1922

## USE OF A CYTOKINE-PRODUCING *LACTOCOCCUS* STRAIN TO TREAT COLITIS

### [0001] CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] This application is a continuation of application PCT/EP99/07800 filed October 6, 1999, designating the United States of America, which itself claims priority from European Patent Application EP 98203529.7, filed on October 20, 1998.

### [0003] TECHNICAL FIELD

[0004] The invention relates generally to medicine, and particularly to an administration strategy for delivering cytokines or cytokine antagonists at the intestinal mucosa. Preferably, the cytokines or cytokine antagonists are acid sensitive anti-inflammatory agents, such as IL10 and/or soluble TNF receptor. These antagonists may be delivered via an oral route. Preferably, inoculation occurs along with a suspension of recombinant *Lactococcus lactis* cells that are engineered to produce the respective proteins.

### [0005] BACKGROUND

[0006] The mammalian immune system is diverse and complex, and includes natural and adaptive immune mechanisms and reactions. The immune system is often described in terms of either "humoral" or "cellular immune" responses. Humoral immunity refers broadly to antibody production and actions by B-cells, while cellular immunity is mediated by cells including T-cells, dendritic cells, neutrophils, monocytes and macrophages. T-cells and B-cells are two categories of lymphocytes.

[0007] One of the mechanisms by which the immune system normally acts and regulates itself includes the production of so-called "cytokines". Cytokines mediate several positive and negative immune responses. Cytokines normally act by binding to a receptor on a target cell. The activity of cytokines can be interfered with in several ways, for example by administration of soluble receptors (extracellular domains of the receptor) or by cytokine analogues or derivatives.

[0008] IL-10 is a cytokine capable of mediating a number of actions and/or effects. It is known that IL-10 is involved in controlling the immune responses of different classes or subsets of Th cells (T-helper cells).



[0009] Inflammatory bowel disease ("IBD") refers to a group of gastrointestinal disorders characterized by a chronic nonspecific inflammation of portions of the gastrointestinal tract. Ulcerative colitis ("UC") and Crohn's Disease ("CD") are the most prominent examples of IBD in humans. They are associated with many symptoms and complications, including growth retardation in children, rectal prolapse, blood in stools (*e.g.*, melena and/or hematochezia), wasting, iron deficiency, and anemia, for example, iron deficiency anemia and anemia of chronic disease or of chronic inflammation. The etiology or etiologies of IBD are unclear. Reference hereto is made in Wyngaarden and Smith (eds.) *Cecil's Textbook of Medicine* (W.B. Saunders Co. 1985), Berkow (ed.) *The Merck Manual of Diagnosis and Therapy* (Merck Sharp & Dohme Research Laboratories, 1982), and *Harrison's Principles of Internal Medicine*, 12<sup>th</sup> Ed., McGraw-Hill, Inc. (1991).

[0010] The incidence of IBD varies greatly with geographic location. A collaborative study was commenced in Europe. It illustrated an incidence of 10.4 per 100,000 for UC and of 5.6 per 100,000 for CD, with 40% and 80% respectively higher incidences for UC and CD in northern centres when compared to those in the south. As both UC and CD are long time afflictions, they correspond to real disturbances in the quality of life. Crohn's disease has a bimodal age distribution of onset, showing striking peaks in incidence at 20 and at 50 years of age. A higher incidence and more grievous disease profile is associated with those that peak at a younger age. This makes CD especially poignant as afflicted adolescents and young adults are virtually deprived of the high expectations of life particularly associated with this age group.

[0011] Ulcerative colitis refers to a chronic, nonspecific, inflammatory, and ulcerative disease having manifestations primarily in the colonic mucosa. It is frequently characterized by bloody diarrhea, abdominal cramps, blood and mucus in the stools, malaise, fever, anemia, anorexia, weight loss, leukocytosis, hypoalbuminemia, and an elevated erythrocyte sedimentation rate ("ESR"). Complications can include hemorrhage, toxic colitis, toxic megacolon, occasional rectovaginal fistulas, and an increased risk for the development of colon cancer.

[0012] Ulcerative colitis is also associated with noncolon complications, such as arthritis, ankylosing spondylitis, sacroileitis, posterior uveitis, erythema nodosum, pyoderma gangrenosum, and episcleritis. Treatment varies considerably with the severity and duration

of the disease. For instance, fluid therapy to prevent dehydration and electrolyte imbalance is frequently indicated in a severe attack. Additionally, special dietary measures are sometimes useful. Medications include various corticosteroids, sulphasalazine and some of its derivatives, and possibly immunosuppressive drugs.

**[0013]** Crohn's Disease shares many features in common with ulcerative colitis. Crohn's Disease is distinguishable in that lesions tend to be sharply demarcated from adjacent normal bowel, in contrast to the lesions of ulcerative colitis which are fairly diffuse. Crohn's Disease predominately afflicts the ileum (ileitis) and the ileum and colon (ileocolitis). In some cases, the colon alone is diseased (granulomatous colitis) and sometimes the entire small bowel is involved (jejunoileitis). In rare cases, the stomach, duodenum, or esophagus are involved. Lesions include a sarcoid-type epithelioid granuloma in roughly half of the clinical cases. Lesions of Crohn's Disease can be transmural including deep ulceration, edema, and fibrosis, which can lead to obstruction and fistula formation as well as abscess formation. This contrasts with ulcerative colitis which usually yields much shallower lesions, although occasionally the complications of fibrosis, obstruction, fistula formation, and abscesses are seen in ulcerative colitis as well.

**[0014]** Treatment is similar for both diseases and includes steroids, sulphasalazine and its derivatives, and immunosuppressive drugs such as cyclosporin A, mercaptopurine and azathioprine. More recently developed treatments, some still in clinical trials, involve systemic administration (by injection) of TNF blocking compounds such as TNF-antibodies or soluble TNF receptor.

**[0015]** IBD represents a genuine problem in public health because of the absence of etiologic treatment. Although many patients are managed successfully with conventional medical therapy, such as anti-inflammatory corticosteroid treatment, most will have recurrent activity of disease, and two-thirds will require surgery.

**[0016]** The cause of inflammatory bowel diseases is unknown. The pathogenesis of CD and UC probably involves interaction between genetic and environmental factors, such as bacterial agents, although no definite etiological agent has been identified so far. The main theory is that abnormal immune response, possibly driven by intestinal microflora, occurs in IBD. It is well established that T-cells play an important role in the pathogenesis. Activated T-cells can produce both anti-inflammatory and pro-inflammatory cytokines. With

this knowledge in hand, IBD can be counteracted in a rational manner. Novel anti-inflammatory therapies, which make use of ~~neutralising~~ neutralizing monoclonal antibodies or anti-inflammatory cytokines, show the possibility to modulate the immune disregulations causative to IBD. A highly prominent and effective new therapy is systemic treatment with anti-TNF monoclonal antibodies as mentioned above. Single intravenous doses, ranging from 5 to 20 mg/kg, of the cA2 infliximab antibody resulted in a drastic clinical improvement in active Crohn's disease. The use of systemically administered recombinant IL-10 in a 7 day by day treatment regime using doses ranging from 0.5 to 25 µg/kg showed reduced Crohn's disease activity scores and increased remission. A number of very promising therapies, either tangling pro-inflammatory cytokines or the establishment of ~~T-cell~~ T-cell infiltrates, are currently emerging from experimental models. All these strategies however require systemic administration. The severe complications of IBD can be seriously debilitating, and eventually may lead to death.

[0017] In U.S. Patent 5,368,854, assigned to Schering Corp., a method is disclosed for using IL-10 to treat inflammatory bowel diseases in mammals. In this method, the cytokine is administered to a mammal having IBD. The administration of IL-10 as described in this reference is parenteral, such as intravascular, preferably intravenous. Such a route of administration for a (human) patient suffering from IBD is, however, not without drawbacks. A much easier and more convenient way would be oral administration of a medicament comprising a cytokine such as IL-10 or a cytokine-antagonist which has a similar therapeutic activity. More importantly, localized release of the therapeutic agent allows for higher efficacy and less unwanted side effects due to systemic activities.

[0018] In WO 97/14806, assigned to Cambridge University Technical Services Ltd., a method is disclosed for delivering biologically active polypeptides and/or antigens by using non-invasive bacteria, such as *Lactococcus*, by intranasally administering the polypeptides to the body, especially at the mucosa.

[0019] However, treating an inflammatory bowel disease such as chronic colitis or Crohn's disease with an acid sensitive cytokine like IL-10, is a very delicate and difficult task to accomplish. Therefore, a system needs to be developed wherein the active compound (*e.g.*, a cytokine or a soluble receptor) is delivered directly at the place where the compound is expected to exert its activity taking into account the acid sensitivity of many cytokines,

particularly IL-10, since, after oral administration, the delivery vehicle needs to pass through the acidic environment of the stomach. Furthermore, various digestive enzymes degrade polypeptides as they pass through the stomach and the gut. Last, but not least, *in situ* administration of the agent may allow one to reach therapeutically effective concentrations difficult to achieve by most systemic routes of administration due to systemic toxicity or other limitations.

## **[0020] SUMMARY OF THE INVENTION**

**[0021]** The invention generally relates to an administration strategy for delivering cytokines, preferably of acid sensitive anti-inflammatory agents, such as IL10 and/or a soluble TNF receptor, via the oral route to the intestinal mucosa. The invention preferably involves inoculation along with a suspension of live recombinant *Lactococcus lactis* cells engineered to produce the respective proteins. For example, mice having a chronic inflammation of the distal colon induced by administration with dextran sulfate sodium (DSS). The treatment, as scored by histological evaluation, clearly showed a regression of the inflammation and disease symptoms. The finding is highly unexpected since, in order to exert activity at the colon following oral administration, the delivery system had to pass the acidic environment of the stomach and the upper part of the small intestine.

## **[0022] BRIEF DESCRIPTION OF THE DRAWINGS**

**[0023]** FIG. 1 depicts the schematic maps of the plasmids used. P1 is the lactococcal P1 promoter as in Waterfield et al, (1995), usp45S is a DNA fragment encoding the secretion signal peptide from the lactococcal Usp45 (van Asseldonck et al, 1990), mil 10 is a DNA fragment encoding the mature part of murine interleukin 10, tr55 is a DNA fragment encoding the soluble part of type 1 TNF receptor, H6 is a fragment encoding 6 histidine residues, Em<sup>r</sup> is the erythromycin selection marker. The DNA sequences of pTREX1 (SEQ ID NO:5), pTINX (SEQ ID NO:6), pTIMIL10 (SEQ ID NO:7), and pT1TR5AH (SEQ ID NO:8) are listed in the enclosed sequence listing and incorporated herein by reference.

**[0024]** FIG. 2 illustrates the protein profile following SDS-PAGE of the culture supernatant of the indicated strains after immunoblot, revealed with anti-murine interleukin 10 (panel A) or anti-murine type 1 TNF receptor and anti-6 His (panel B) antisera.

[0025] FIG. 3 is a bar graph depicting the average of colon length of groups of mice in which: a) chronic colitis had been induced with DSS, b) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain ~~MG1363pT1MIL10~~ MG1363(pTREX1) was orally administered, c) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain ~~MG1363pT1MIL10~~ MG1363(pT1TR5AH) was orally administered and d) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain ~~MG1363pT1MIL10~~ MG1363(pT1MIL10) was orally administered.

[0026] FIG. 4 is a bar graph depicting the average of epithelial damage score in the distal colon of groups of mice in which: a) chronic colitis had been induced with DSS, b) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain ~~MG1363pT1MIL10~~ MG1363(pTREX1) was orally administered, c) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain ~~MG1363pT1MIL10~~ MG1363(pT1TR5AH) was orally administered and d) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain ~~MG1363pT1MIL10~~ MG1363(pT1MIL10) was orally administered.

[0027] FIG. 5 is a bar graph depicting the average of inflammatory infiltrate score in the distal colon of groups of mice in which: a) chronic colitis had been induced with DSS, b) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain ~~MG1363pT1MIL10~~ MG1363(pTREX1) was orally administered, c) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain ~~MG1363pT1MIL10~~ MG1363(pT1TR5AH) was orally administered and d) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain ~~MG1363pT1MIL10~~ MG1363(pT1MIL10) was orally administered.

[0028] FIG. 6 shows representative sections of mice distal colon stained with haematoxylin and eosin. Specifically, the picture shown illustrates normal tissue in untreated animals.

[0029] FIG. 7 shows representative sections of mice distal colon stained with haematoxylin and eosin. Specifically, the picture shown illustrates animals pretreated with DSS to acquire chronic colitis.

[0030] FIG. 8 shows representative sections of mice distal colon stained with haematoxylin and eosin. Specifically, the picture shown illustrates animals pretreated with

DSS to acquire chronic colitis to which *L. lactis* strain ~~MG1363pT1MIL10~~ MG1363(pT1MIL10) was subsequently orally administered.

[0031] FIG. 9 shows representative sections of mice distal colon stained with haematoxylin and eosin. Specifically, the picture shown illustrates animals pretreated with DSS to acquire chronic colitis to which *L. lactis* strain ~~MG1363pTREX1~~ MG1363(pTREX1) was subsequently orally administered.

[0032] FIG. 10 is a graph illustrating statistical evaluation of the histology. The colon sections were randomly numbered and interpreted blind. Scores from individual mice were subsequently decoded and the regrouped numbers were analyzed statistically. The DSS colitis panel shows histological sum scores for the distal colon of blank mice and of mice induced with DSS to acquire chronic colitis, either untreated or treated with *L. lactis* cultures. The score is a sum of scores for epithelial damage and lymphoid infiltrate, both ranging between 0 and 4. Groups of mice (n = 10) were alternatively treated with MG1363, MG1363(pTREX1) or MG1363(pT1MIL10) (= IL-10) for two (= 2w) or four (= 4w) weeks. Some of the cultures were irradiated with uv (= + uv) prior to inoculation, which reduced cell viability over 10<sup>6</sup> times. The IL-10-/- colitis panel shows histological sum scores of groups (n = 5) of 7 week old untreated, TREX treated and IL-10 treated female 129 Sv/Ev IL-10-/- mice. The histological score is a sum of the degree of inflammation in the proximal, middle and distal colon, all ranging between 0 and 4. Error bars represent s.e.m.

[0033] FIG. 11 is a graph that shows the representation of bacterial viability after irradiation as measured at OD<sub>600</sub>.

#### [0034] DETAILED DESCRIPTION OF THE INVENTION

[0035] In order to achieve the recovery of a patient suffering from an IBD, it is necessary to restore the damaged cells and the organ comprising the damaged cells, for instance the colon. The solution to the above described technical problem is achieved by providing the embodiments characterized below.

[0036] According to the invention, cytokine-producing Gram-positive bacterial strain or a cytokine antagonist producing Gram-positive bacterial strain is used for the preparation of a medicament to treat inflammatory bowel disease.

[0037] The cytokine or cytokine antagonist to be produced by the bacterial host strain is, for instance, IL-10, a soluble TNF receptor or a cytokine analogue such as the IL-12 derived p40 homodimer (an IL-12 antagonist), an Interferon- $\gamma$ -antagonist, an IL-1 antagonist or a virus-coded cytokine analogue such as EBV BCRF1 (Baer et al., 1984), whereas the Gram-positive bacterial strain preferably is a *Lactococcus* species, and more preferably, a *Lactococcus lactis*.

[0038] Other Gram-positive bacterial strains to be used for the purpose of the current invention are *Bacillus subtilis*, *Streptococcus gordonii*, *Staphylococcus xylosus*, or a *Lactobacillus* species, such as *L. bulgaricus*, *L. salivarius*, *L. casei*, *L. helveticus*, *L. delbrueckii* or *L. plantarum*.

[0039] The inflammatory bowel diseases such as a chronic colitis, Crohn's disease and ulcerative colitis can be treated according to the invention with an appropriate dosage of the active cytokine compound, preferably IL-10 or soluble TNF receptor. The treatment unexpectedly restores the diseased colon to an apparently normal and healthy state.

[0040] IL-10 can be administered alone or in combination with at least one additional therapeutic agent. Examples of such additional therapeutic agents include corticosteroids, sulphasalazine, derivatives of sulphasalazine, immunosuppressive drugs such as cyclosporin A, mercaptopurine, azathioprine, and another cytokine. The co-administration can be sequential or simultaneous. Co-administration generally means that the multiple (two or more) therapeutics are present in the recipient during a specified time interval. Typically, if a second agent is administered within the half-life of the first agent, the two agents are considered co-administered.

[0041] The invention disclosed herein thus concerns a localized delivery of IL-10 through *in situ* synthesis by recombinant *L. lactis*. As a result thereof, the inflammation is reduced by 50% in chronic colitis induced with DSS, and prevents the onset of colitis in IL-10 -/- 129 Sv/Ev mice. So the method is equally efficient in comparison to powerful, well-established and accepted therapies relying on the systemic administration of anti-inflammatory proteins.

[0042] The vector, *L. lactis*, is a Gram positive food grade organism which is believed to be totally harmless. It is a non-colonizing micro-organism. Accurate dosage and timing during treatment, shown here to be of great importance, can thus easily be obtained.

[0043] The critical requirement for viability of the vector is shown in the current invention. This indicates the need for *in situ* synthesis of IL-10. The vector is indeed capable of achieving this by showing *de novo* synthesis of IL-10 in the colon.

[0044] An efficient novel concept for protein-based treatment in the intestinal tract is herein disclosed. The treatment can be given by the oral route, which is by far the most desirable for pharmacological formulations. It can exert effects up to the distal colon using a compound with intrinsic sensitivity for the route used. This method bypasses the need for systemic administration. It opens the possibility for the localized delivery of substances, which are unstable or difficult to produce in high quantities. Intrinsically, it is very cost effective. In comparison to systemic delivery, the method may provide for sustained and localized presence of IL-10 at concentrations higher than desirable or even achievable with systemic delivery, especially with regard to latent side effects.

[0045] Some terms used in the current description are, for sake of clarity, explained hereafter.

[0046] Generally, the term “symptoms” refers to any subjective evidence of disease or of a patient’s condition. This includes evidence as perceived by the patient. Examples of symptoms of IBD include diarrhea, abdominal pain, fever, melena, hematochezia, and weight loss.

[0047] The term “signs” refers generally to any objective evidence of a disease or condition, usually as perceived by an examining physician or features which would reveal themselves on a laboratory evaluation or other tests such as an ultrasonic study or a radiographic test. Some examples of signs of IBD include abdominal mass, glossitis, aphthous ulcer, anal fissure, perianal fistula, anemia, malabsorption, and iron deficiency. Occasionally, signs and symptoms overlap. For example, the patient complains of blood stools (a symptom), and a laboratory test of a stool sample is positive for blood (a sign).

[0048] The phrase “appropriate dosage” or “effective amount” means an amount or dosage sufficient to ameliorate a symptom or sign of an autoimmune condition or of an undesirable or inappropriate inflammatory or immune response. An effective amount for a particular patient may vary depending on factors such as the condition being treated, the overall health of the patient, the method, route and dose of administration and the severity of the side affects.



[0049] With “cytokine” is meant a polypeptide factor produced transiently by a range of cell types, acting usually locally, and activating the expression of specific genes by binding to cell surface receptors.

[0050] With “antagonist” is meant a compound that binds to but does not activate receptors, and hence inhibits the action of an agonist competitively.

[0051] “Agonists” are compounds that bind to and activate receptors (e.g., endogenous ligands such as hormones and neurotransmitters, chemically synthesized compounds, natural products like alkaloids).

[0052] The invention is further explained by the following methods used in the current invention.

**[0053] Culture media**

[0054] GM17 is M17 (Difco, St. Louis, MO, US) supplemented with 0.5 w/v % of glucose. GM17E is GM17 supplemented with 5 µg/ml of erythromycin. BM9 contains per liter 6 g of Na<sub>2</sub>HPO<sub>4</sub>, 3 g of KH<sub>2</sub>PO<sub>4</sub>, 1 g of NH<sub>4</sub>Cl, 0.5 g of NaCl, 2 mmol of MgSO<sub>4</sub>, 25 mmol of NaHCO<sub>3</sub>, 25 mmol of Na<sub>2</sub>CO<sub>3</sub>, 0.1 mmol of CaCl<sub>2</sub>, 5 g of glucose and 5 g of casitone (Difco). BM9E is BM9 supplemented with 5 µg/ml of erythromycin.

**[0055] Recombinant DNA techniques.**

[0056] PCR amplification of DNA was performed with VENT polymerase and using conditions recommended by the manufacturer. DNA modifying enzymes and restriction endonucleases were used under standard conditions and in the buffers recommended by the manufacturers. General molecular cloning techniques and the electrophoresis of DNA and proteins were carried out essentially as described (Sambrook et al., 1990). *L. lactis* was transformed by electroporation of cells grown in the presence of glycine (Wells et al., 1993).

[0057] The plasmid pT1MIL10 (FIG. 1) was constructed by subcloning a PCR fragment, obtained with the primers (CAGTACAGCCGGAAGACAAT (SEQ ID NO:1) and GCACTAGTTAGCTTTTCATTTTGAT (SEQ ID NO:2)) and performed on a cDNA clone containing mIL10 coding sequence. For the design of this strategy, we made use of the mIL10 cDNA sequence as given in EMBL acc. nr. M37897. By utilizing the above-mentioned primers, the mIL10 fragment could be subcloned as a blunt – SpeI fragment, after treatment with kinase and SpeI, in the NaeI-SpeI opened plasmid pT1NX (FIG. 1), which is a pTREX1 derivative (Wells and Schofield in: Lactic Acid Bacteria: current advances in metabolism,

genetics and applications. F. Bozoglu & R. Bibek, Eds., *Nato ASI Series H*, Vol.98, p. 37. Springer-Verlag, 1996.)

**[0058]** The plasmid pT1TR5AH (FIG. 1) was constructed by subcloning a PCR fragment, obtained with the primers (CTGGTCCCTTCTCTTGGTGAC (SEQ ID NO:3) and CCACTAGTCTATTAATGATGATGATGATGATGCGCAGTACCTGAGTCCTGGGG (SEQ ID NO:4)) and performed on a cDNA clone containing sTNFr55 coding sequence. In designing this strategy, we made use of the TNFr55 cDNA sequence as given in EMBL acc. nr. L26349. By utilizing the above-mentioned primers, the sTNFr 55 fragment was provided with a 6his tag at the 3' end and could be subcloned as a blunt – SpeI fragment, after treatment with kinase and SpeI, in the NaeI-SpeI opened plasmid pT1NX.

**[0059]** Both plasmids code, downstream from the lactococcal P1 promoter, for fusion genes between the secretion leader from Usp45 (Van Asseldonk et al., *Gene*, 95, 155-160, 1990) and mIL10 and sTNFr 55, respectively. Upon secretion, the leader sequence is cleaved off.

#### **[0060] Identification of recombinant proteins**

**[0061]** Recombinant mIL10 and msTNFr 55 could be observed in the supernatant of cultures of ~~MG1363[pT1MIL10]~~ MG1363(pT1MIL10) and ~~MG1363[pT1TR5AH]~~ MG1363(pT1TR5AH), respectively (FIG. 2). For this test, 5 ml aliquots of the cultures were extracted with 2 ml phenol and the proteins were subsequently prepared from the organic phase by precipitation with 10 ml of ethanol. A part of the precipitate, equivalent to 1 ml of culture supernatant, was subjected to SDS-15% PAGE and immunoblotting. Culture samples were taken at relevant times in the growth phase of the bacteria, as described below.

**[0062]** The culture supernatant of ~~MG1363[pT1MIL10]~~ MG1363(pT1MIL10) contained, on average, 1 µg/ml of murine IL10. Murine IL-10 activity of the supernatant was measured using a murine mast cell line MC/9 (Thompson-Snipes, L. et al., *J. Exp. Med.* 173, 507, 1991). Human IL-10 binds to murine IL-10R as was demonstrated by transfection experiments (Ho, A.S.Y et al., *PNAS* 90, 11267, 1993; Liu, Y. et al., *J.Immunol.* 152, 1821, 1994). 1 U/ml of IL-10 is defined as the amount of IL-10 that is able to inhibit 50% the level of IFN-gamma production of conA activated splenocytes (Fiorentino, D.F. et al., *J.Exp.Med.* 170, 2081, 1989). The ED50 for this effect is typically 0.3-0.6 ng/ml. When measured along with a standard of known activity (Biosource International, CA) the ~~MG1363[pT1MIL10]~~

MG1363(pT1MIL10) culture supernatant revealed an activity of approximately 8000 U/ml. Berg et al. (*J. Clin. Invest* 98, 1010-1020) report a specific activity of approximately  $1.0 \times 10^7$  U/mg for recombinant mIL10. From these considerations, and taking into account the variations in the method used, we concluded that the recombinant mIL10, present in the ~~MG1363(pT1MIL10)~~ MG1363(pT1MIL10) culture supernatant, displayed full biological activity. No IL10 activity could be detected in the supernatant of the control cultures, MG1363 or ~~MG1363(pTREX1)~~ MG1363(pTREX1).

[0063] The culture supernatant of strain ~~MG1363(pT1TR5AH)~~ MG1363(pT1TR5AH) contained, on average, 200 ng/ml msTNFr 55. Loetscher et al. (1991) showed that complete inhibition of TNF cytotoxic activity by sTNFr 55 was only obtained from a molar ratio of 1000 : 1 of sTNFr 55 to TNF and higher. The soluble recombinant TNFr 55 which had been recovered from the culture supernatant of MG1363(pT1TR5AH) showed an equal inhibitory effect on TNF as had been reported for the indigenous product. This was demonstrated by mixing up and thus competing out a titration series of TNF with a titration series of recombinant sTNFr and measuring TNF activity in a cytotoxicity assay as described (Espevik, T and Nissen-Meyer, 1986).

**[0064] Pretreatment of the mice**

[0065] For the induction of chronic colitis, mice were pretreated as described by Kojouharoff et al. *Clin Exp Immunol* 107, 353, 1997. Six to eight weeks old female Balb/c mice received four cycles of treatment with DSS. Each cycle consisted of 5% DSS in the drinking water for 7 days, followed by a 10-day interval during which they received normal drinking water. Four to six weeks after completion of the last DSS cycle, mice were treated with the *L. lactis* strains as indicated.

[0066] The invention is further explained by the use of the following illustrative examples.

[0067] Examples

[0068] Example 1

[0069] Treatment of the mice with live *L. lactis*

[0070] Storage of expression strains.

[0071] Freshly streaked cultures of the *L. lactis* expression strains were inoculated in 10 ml of GM17 or GM17E depending on the absence or presence of an expression plasmid and grown overnight at 30°C. The overnight cultures were diluted 1/100 in fresh GM17 or GM17E and pregrown for 3 hours at 30°C. The cells were harvested by centrifugation and resuspended in BGM9 or BGM9E, depending on the presence of plasmids. These cultures were grown for 5 hours at 30°C. The protein profile of these cultures was analyzed by performing Western immunoblotting on an equivalent of 1 ml of culture supernatant using either antiserum directed towards sTNFr 55 or IL10 respectively. The protein profile of sTNFr 55 and IL10 is shown in the appropriate lanes (FIG. 2). 5 ml of the original GM17 or GM17E overnight cultures were supplemented with 5 ml of glycerol and stored at -20°C. These stocks were used as starter material for several experiments. Protein analysis throughout a series of individual experiments showed that a high degree of reproducibility in the production of the recombinant proteins could be obtained by this procedure.

[0072] Weeks 1 and 2

[0073] Stock solutions of *L. lactis* strains were diluted 1/200 in 10 ml GM17 or GM17E and grown overnight at 30°C. The cells were harvested by centrifugation and resuspended in 1 ml BM9 or BM9E. Control, healthy mice and mice with induced colitis were inoculated on a daily basis with 100 µl aliquots of these cell suspensions.

[0074] Weeks 3 and 4

[0075] Stock solutions of *L. lactis* strains were diluted 1/200 in 10 ml GM17 or GM17E and grown overnight at 30°C. These cultures were diluted 1/25 in 10 ml of BM9 or BM9E and grown for 3 hours at 30°C. Aliquots of 200 µl were intragastrically (peroral) administered into mice on a daily basis.

[0076] Example 2

[0077] Determination of histological score

[0078] Histological score was determined essentially as described by Kojouharoff et al. I 107, 353, 1997.

[0079] Mice were ~~euthanised~~ euthanized by cervical dislocation. The colon was removed and washed with PBS. The distal third of the colon was cut longitudinally, laid on filter paper and fixed with 10% formalin in PBS overnight. Sections of the paraffin-embedded material were made longitudinally. Three 3- $\mu$ m sections were cut at an intermediate distance of 200  $\mu$ m. The sections were stained with haematoxylin-eosin. Histological analysis was performed in blind fashion. Mice were scored individually, and each score represented the mean of three sections.

[0080] Histology was scored as follows:

[0081] Infiltration: 0, no infiltrate; 1, infiltrate around crypt bases; 2, ~~Infiltrate~~ infiltrate reaching to L. muscularis mucosae; 3, extensive infiltration reaching the L. muscularis mucosae and thickening of the mucosa with abundant oedema; 4, infiltration of the L. submucosa.

[0082] Epithelial damage: 0, normal morphology; 1, loss of goblet cells; 2, loss of goblet cells in large areas; 3, loss of crypts; 4, loss of crypts in large areas and/or foci of polyploid regeneration.

[0083] Colonic length was measured immediately after dissection and placement on a paper towel. The pathology of chronic colitis is, amongst other parameters, ~~characterised~~ characterized by a decrease in length of the colon and by epithelial damage and infiltration of lymphocytes to a more or less substantial extent. FIG. 3 clearly shows an increase in colon length after the treatment of the inflamed mice with ~~MG1363[pT1MIL10]~~ MG1363(pT1MIL10) and, although to a lesser extent, after the treatment of the mice with ~~MG1363[pT1TR5AH]~~ MG1363(pT1TR5AH).

[0084] FIGS. 4 and 5 show the onset of recovery from chronic colitis, in which mice treated with MG1363(pT1MIL10) appear to improve more extensively than those mice which had been treated with ~~MG1363[pT1TR5AH]~~ MG1363(pT1TR5AH).

[0085] FIG. 4 shows the histological score of epithelial damage whereas FIG. 5 shows inflammatory infiltrate, both determined as described previously.

[0086] FIGS. 6-9 shows the histology of normal tissue, compared to inflamed and treated tissue.

[0087] In the normal histology, one can observe a continuous array of crypts of equal length. In the crypts, numerous goblet cells can be observed. A low number of lymphocytes

is present in the mucosa. No lymphocytes are present in the submucosa. In the inflamed tissue, one can see the disappearance of the ~~organised~~ organized crypt structures, ranging from differences in length to complete absence of structure. Also, in the relicts of the crypts no goblet cells are present. One can observe a large increase of the thickness of the mucosa due to a massive infiltration of lymphocytes. The lymphocytes tend to form ulcerations. In severe cases, infiltration of lymphocytes can also be observed in the submucosa. The epithelium, however, remains intact. The negative control of treatment with MG1363( pTREX1) shows a pathology reminiscent of that of heavily inflamed tissue. Mice treated with MG1363 (pT1MIL10) show an almost complete restitution of the normal histology, revealing only slight remainders of infiltrating lymphocytes in the mucosa. Mice treated with ~~MG1363(pT1TR5AH)~~ MG1363(pT1TR5AH) show an intermediate degree in pathology.

**[0088]** FIG. 10 shows the statistic evaluation of histological scores obtained from individual mice following treatment with the indicated *L. lactis* strains (group size = 10). The score was recorded after blind interpretation of slides from the distal colon as described (Kojouharoff et al., 1997). Each mouse was interpreted according to 3 longitudinal slides, equally spaced over the circumference of the colon. Both lymphoid infiltrate and epithelial damage were rated from 0 to 4 points and values for both parameters were summed for every mouse. Normal blank mice showed a histological score of 1 point. The mice induced for colitis are slightly over 5 points. All of the control groups for *L. lactis* treatment fluctuate around this number, with possibly a slightly higher tendency in some groups. The mice treated for 14 days with mIL-10 producing *L. lactis*, followed by 14 days of recovery however show an average of approximately 3 points. This is a decrease of nearly 50% in the pathology when measured against the difference between untreated and blank control groups. The reduction is significant ( $p = 0.0151$ ).

### **[0089]** Example 3

**[0090]** Due to the culture conditions used, a minor amount (40 ng) of mIL-10 is present in the supernatant of the inoculation suspension. To investigate whether this IL-10 brings about the observed reduction in the histological score we included treatment with UV killed IL-10 producer strains. These cultures were UV irradiated immediately prior to the inoculation. FIG. 11 shows that irradiation reduced the bacterial viability to less than  $1 \text{ in } 10^6$  cfu so that no further accumulation of IL-10 was observed. This was not associated with cell

lysis since no drop in OD<sub>600</sub> was observed and no IL-10 precursor could be detected in the culture supernatant. The irradiation does not affect IL-10 bioactivity. Diseased mice treated for 2 or 4 weeks with the UV dispatched cultures show no difference in colon histology when compared to any of the control groups positive for enterocolitis. The fate of the residual IL-10 in the inoculation medium is most likely denaturation and breakdown in the stomach and duodenum. The acidity of the stomach, prior at pH 1.5, rises to pH6 immediately after inoculation. After 5 minutes a pH of 4 is reached, which further drops from 3.5 to 2.5 in the interval between 30 and 60 minutes after inoculation. IL-10 detected in the stomach 5 minutes after inoculation rapidly decreases in concentration and was only found in trace amounts in the duodenum at 30 minutes after inoculation. At later time-points, no IL-10 was detected here nor in the jejunum or ileum.

**[0091] Example 4**

**[0092]** Seven serial inoculations of  $3.4 \times 10^9$  cfu of MG1363(pT1MIL10) were given to 129 Sv/Ev IL-10<sup>-/-</sup> mice, thereby respecting 1 hour intervals. The intestine was prepared out 30 minutes after the last inoculation and divided in the morphologic compartments. Immediately the tissues were ~~homogenised~~ homogenized in PBS with 1% BSA and 0.05% NaN<sub>3</sub>. Cfu of MG1363(pT1MIL10) were determined as  $7 \times 10^6$  in the stomach,  $2.6 \times 10^8$  in the duodenum,  $2.8 \times 10^7$  in the jejunum,  $4 \times 10^8$  in the ileum,  $8.4 \times 10^8$  in the caecum and  $7 \times 10^8$  in the colon. We have detected 70 ng of soluble IL-10 in the colon homogenate. None of the upstream compartments showed any IL-10 content. From this it is concluded that recombinant *L. lactis* can actively produce IL-10 in the colon.

**[0093] Example 5**

**[0094] Prevention of enterocolitis in IL10<sup>-/-</sup> mice**

**[0095]** The capacity of the approach described above was tested to prevent the onset of colitis in 129 Sv/Ev IL10<sup>-/-</sup> mice. These mice spontaneously developed a generalized enterocolitis in the frame between three and eight weeks of age (Kuhn et al., *Cell*,1993;75:263-274). Inflammatory changes first appear in the cecum, ascending and transverse colon of 3-wk-old mutants. Progressive disease in aging IL10<sup>-/-</sup> mice was characterized by an increased number of multifocal inflammatory cell infiltrates composed of mononuclear cells and neutrophils accompanied by moderate epithelial hyperplasia and slight mucin depletion from goblet cells. Small epithelial erosions and crypt abscesses were

occasionally present and inflammation rarely involved the submucosa. IL10<sup>-/-</sup> mice used in our studies showed a less severe inflammation as described due to “clean” rather than “conventional” conditions of our animal facility.

[0096] When these mice are treated from week 3 on, for 6 to 8 weeks with either anti IFN- $\gamma$  or anti-IL-12 colitis can be prevented (Rennick et al., *J-Leukoc-Biol.*, 1997 Apr; 61(4):389-396). We treated 3 weeks old mice by daily intra-gastric inoculation with IL-10 producing *L. lactis*. The mice were treated for 4 weeks with either mid-log or end-log cultures whilst an untreated group was kept under identical conditions. FIG. 10 shows histological scores obtained as described (Berg et al., *J-Clin-Invest*; 1996, Aug 15; 98(4):1010-1020), with the exception that we did not examine the caecum. The nontreated mice show a mean histological score of approximately 4.5 points. This fits well with reported data, provided one takes into account the contribution of the caecal scores in these values and the slight age difference. The group of mice treated with MG1363(pT1MIL10) shows a mean histological score of 1.5 points which is only slightly over values reported for 3 week old mice (Berg et al., *J-Clin-Invest*; 1996, Aug 15; 98(4):1010-1020). As it is the sum of 3 values ranging from 0 to 4 points, this is considered as a very low score. From these data it is clear that the development of colitis can be prevented by this treatment.



- [0098] Wells J.M., & Schofield, K.M. Cloning and expression vectors for lactococci From: Lactic Acid Bacteria (eds Bozoglu B., and Ray, B.) *NATO ASI Series H* 98: 37-63 Springer-Verlag, Berlin, Heidelberg (1996).
- [0099] Kojouharoff, G., Hans, W., Obermeier, F., Mannel, D.N., Andus, T., Scholmerich, J., Gross, V. & Falk, W. Neutralization of tumour necrosis factor (TNF) but not of IL-1 reduces inflammation in chronic dextran sulphate sodium-induced colitis in mice. *Clin. Exp. Immunol.* 107, 353 - 358, 1997.
- [0100] Van Asseldonk, M., Rutten, G., Oteman, M., Siezen, R.J., de Vos, W.M. and Simons, G. Cloning of *usp45*, a gene encoding a secreted protein from *Lactococcus lactis* subsp. *lactis* MG1363. *Gene*, 95, 155-160 (1990).
- [0101] Sambrook, J., Fritsch, E.F., and Maniatis T. Molecular cloning-a laboratory manual. *Cold Spring Harbor Laboratory*, New York (1990).
- [0102] Wells, J.M., Wilson, P.W., and Le Page, R.W.F. Improved cloning vectors and transformation procedure for *Lactococcus lactis*. *J. Appl. Bacteriol.* 74, 629-636 (1993).
- [0103] Schlaak, J.F., Schmitt, E., Huls, C., Meyer zum Buschenfelde, K.H. & Fleischer, B. A sensitive and specific bioassay for the detection of human interleukin-10. *J. Immunol. Methods* 168, 49-54, 1994.
- [0104] Thompson-Snipes, L., Dhar, V., Bond, M.W., Mosmann, T.R., Moore, K.W. & Rennick, DM Interleukin 10: a novel stimulatory factor for mast cells and their progenitors. *J. Exp. Med.* 173, 507-10, 1991.

[0105] Ho, A., S., Y., Liu, Y., Khan, T., A., Hsu, D., H., Bazan, J., F. & Moore, K., W. A receptor for interleukin 10 is related to interferon receptors. *PNAS* 90(23): 11267-11271 (1993)

[0106] Liu, Y., Wei, S., H., Y., Ho, A., S., Y., De Waal-Malefyt, R. & Moore, K., W. Expression cloning and characterization of a human IL-10 receptor. *Journal of Immunology* 152(4): 1821-1829 (1994)

[0107] Fiorentino, D.F., Bond, M.W. & Mosmann, T.R. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J-Exp-Med.* 170, 2081-95, 1989.

[0108] Waterfield, N.R. et al., The isolation of lactococcal promoters and their use in investigating bacterial luciferase synthesis in *lactococcus lactis*. *Gene*, 165, 9-15 (1995).

[0109] Baer, R. et al., DNA sequence and expression of the B95-8 Epstein-Barr virus genome. *Nature*, 130, 207-211 (1984).

## ABSTRACT

An administration strategy for the delivery at the intestinal mucosa of cytokines or cytokine antagonists, preferably of acid sensitive anti-inflammatory agents, such as IL10 and/or soluble TNF receptor via the oral route. Preferably, inoculation occurs along with a suspension of recombinant *Lactococcus lactis* cells, which had been engineered to produce the respective proteins.

## SEQUENCE LISTING

<110> Steidler, Lothar

Remaut, Erik

Fiers, Walter

<120> USE OF A CYTOKINE-PRODUCING LACTOCOCCUS STRAIN TO TREAT COLITIS

<130> 2676-4779US

<150> PCT/EP99/07800

<151> 1999-10-06

<150> EP 98203529.7

<151> 1998-10-20

<160> 8

<170> PatentIn version 3.0

<210> 1

<211> 21

<212> DNA

<213> Artificial

<220>

<223> Description of Artificial Sequence: primer used for obtaining the  
plasmid pT1MIL1

<400> 1

cagtacagcc gggaagacaa t

21

<210> 2

<211> 25

<212> DNA

<213> Artificial

<220>

<223> Description of Artificial Sequence: primer used for obtaining the  
plasmid pT1MIL1

<400> 2

gcactagtta gcttttcatt ttgat

25

<210> 3

<211> 21

<212> DNA

<213> Artificial

<220>

<223> Description of Artificial Sequence: primer used for obtaining the  
plasmid pT1TR5A

<400> 3  
ctggtccctt ctcttggtga c

21

<210> 4

<211> 53

<212> DNA

<213> Artificial

<220>

<223> Description of Artificial Sequence: primer used for obtaining the  
plasmid pT1TR5A

<400> 4  
ccactagtct attaatgatg atgatgatga tgcgcagtag ctgagtcctg ggg 53

<210> 5

<211> 5230

<212> DNA

<213> Artificial

<220>

<223> Description of Artificial Sequence: plasmid pTREX1

<400> 5  
gaattcgatt aagtcattt accctcttta ttagttttt cttataatct aatgataaca 60

ttttataat taatctataa accatatccc tctttggaat caaaatttat tatctactcc 120

ttttagata tgttataata caagtatcag atctgggaga ccacaacggt tteccactag 180

aaataatttt gtttaacttt agaaaggaga tatacgcatg caggatatct ctagaatgga 240

tccggctgct aacaaagccc gaaaggaagc tgagttggct gctgccaccg ctgagcaata 300  
 actagcataa ccccttgggg cctctaaacg ggtcttgagg ggtttttgc tgaaaggagg 360  
 aactatatcc ggatgacctg caggcaagct ctagaatcga tacgatttg aagtggcaac 420  
 agataaaaaa aagcagtta aaattgttc tgaacttta aaacaagcaa atacaatcat 480  
 tgtcgcaaca gatagcgaca gagaaggcga aaacattgcc tggtcgatca ttcataaagc 540  
 aaatgccttt tctaaagata aaacgtataa aagactatgg atcaatagtt tagaaaaaga 600  
 tgtgatccgt agcggtttc aaaattgca accaggaatg aattactatc ccttttatca 660  
 agaagcgcaa aagaaaaacg aaatgataca ccaatcagtg caaaaaaaga tataatggga 720  
 gataagacgg ttcgtgttcg tgctgacttg caccatatca taaaaatcga aacagcaaag 780  
 aatggcggaa acgtaaaaga agttatggaa ataagactta gaagcaaact taagagtgtg 840  
 ttgatagtgc agtatcttaa aattttgtat aataggaatt gaagttaa tagatgctaa 900  
 aaatttgtaa ttaagaagga gtgattacat gaacaaaaat ataaaatatt ctcaaaactt 960  
 tttacgagt gaaaaagtac tcaaccaa ataaaaacaa ttgaatttaa aagaaaccga 1020  
 taccgtttac gaaattggaa caggtaaagg gcatttaacg acgaaactgg ctaaaataag 1080  
 taaacaggta acgtctattg aattagacag tcatctatc aactatcgt cagaaaaatt 1140  
 aaaactgaat actcgtgtca ctttaattca ccaagatatt ctacagttc aattccctaa 1200  
 caaacagagg tataaaattg ttgggagtat tccttaccat ttaagcacac aaattattaa 1260  
 aaaagtgtt ttgaaagcc atgcgtctga catctatctg attgttgaag aaggattcta 1320  
 caagegtacc ttgatattc accgaacact agggttgctc ttgcacactc aagtctgat 1380  
 tcagcaattg cttaaagtgc cagcggaatg ctttcacct aaacaaaag taaacagtgt 1440  
 cttataaaaa cttaccgcc ataccacaga tgtccgat aaatattgga agctatatac 1500  
 gtactttgt tcaaatggg tcaatcgaga atatcgtaa ctgtttacta aaaatcagtt 1560  
 tcatcaagca atgaaacacg ccaaagtaa caatttaagt accgttactt atgagcaagt 1620

attgtctatt ttaatagtt atctattatt taacgggagg aaataattct atgagtcgct 1680  
 ttgttaaatt tggaaagtta cacgttacta aagggaatgt agataaatta ttaggtatac 1740  
 tactgacagc ttccaaggag ctaaagaggt ccctagcgct cttatcatgg ggaagctcgg 1800  
 atcatatgca agacaaaata aactcgcaac agcacttgga gaaatgggac gaatcgagaa 1860  
 aaccctcttt acgctggatt acatatctaa taaagccgta aggagacggg ttcaaaaagg 1920  
 tttaaataaa ggagaagcaa tcaatgcatt agctagaact atatttttg gacaacgtgg 1980  
 agaatttaga gaacgtgctc tccaagacca gttacaaaga gctagtgcac taaacataat 2040  
 tattaacgct ataagtgtgt ggaacactgt atatatggaa aaagccgtag aagaattaaa 2100  
 agcaagagga gaatttagag aagatttaat gccatatgcg tggccgttag gatgggaaca 2160  
 tatcaatfff cttggagaat acaaattga aggattacat gacactgggc aaatgaattt 2220  
 acgtccttta cgtataaaag agccgtttta ttcttaatat aacggctctt tttatagaaa 2280  
 aaatccttag cgtgggtttt ttccgaaatg ctggcggtag cccaagaatt agaatgagt 2340  
 agatcaaatt attcacgaat agaatcagga aaatcagatc caaccataaa aacactagaa 2400  
 caaattgcaa agttaactaa ctcaacgcta gtagtggatt taatcccaa tgagccaaca 2460  
 gaaccagagc cagaaacaga atcagaacaa gtaacattgg atttagaaat ggaagaagaa 2520  
 aaaagcaatg acttcgtgtg aataatgcac gaaatcgttg cttattttt tttaaagcg 2580  
 gtatactaga tataacgaaa caacgaactg aatagaaacg aaaaagagc catgacacat 2640  
 ttataaaatg ttgacgaca tttataaat gcatagcccg ataagattgc caaaccaacg 2700  
 cttatcagtt agtcagatga actcttcctt cgtaagaagt tatttaatta actttgttg 2760  
 aagacggtat ataaccgtac tatcattata tagggaaatc agagagtttt caagtatcta 2820  
 agctactgaa ttaagaatt gttaagcaat caatcgaaa tcgtttgatt gcttttttg 2880  
 tattcattta tagaaggtgg agtttgatg aatcatgatg aatgtaaaac ttatataaaa 2940  
 aatagtttat tggagataag aaaattagca aatatctata cactagaaac gttaagaaa 3000



gagttagaaa agagaaatat ctacttagaa acaaaatcag ataagtattt ttcttcggag 3060  
 ggggaagatt atatatataa gttaatagaa aataacaaaa taatttattc gattagtga 3120  
 aaaaaattga cttataaagg aaaaaaatct tttcaaaac atgcaatatt gaaacagttg 3180  
 aatgaaaaag caaaccaagt taattaaaca acctatttta taggatttat aggaaaggag 3240  
 aacagctgaa tgaatatccc tttgttgta gaaactgtgc ttcatgacgg cttgttaaag 3300  
 taaaattta aaaatagtaa aattcgctca atcactacca agccaggtaa aagcaaaggg 3360  
 gctatttttg cgtatcgtc aaaatcaagc atgattggcg gtcgtggtgt tgttctgact 3420  
 tccgaggaag cgattcaaga aaatcaagat acatttacac attggacacc caacgtttat 3480  
 cgttatggaa cgtatgcaga cgaaaaccgt tcatacacga aaggacattc tgaaaacaat 3540  
 ttaagacaaa tcaatacctt ctttattgat ttgatattc acacggcaaa agaaactatt 3600  
 tcagcaagcg atattttaac aaccgctatt gatttaggtt ttatgcctac tatgattac 3660  
 aaatctgata aaggttatca agcatatttt gtttagaaa cgccagtcta tgtgacttca 3720  
 aaatcagaat ttaaatctgt caaagcagcc aaaataattt cgaaaatat ccgagaatat 3780  
 tttgaaagt ctttgccagt tgatctaacg tgtaatcatt ttggtattgc tcgcatacca 3840  
 agaacggaca atgtagaatt tttgatcct aattaccgtt attctttcaa agaattggcaa 3900  
 gattggtctt tcaaacaaac agataataag ggctttactc gttcaagtct aacggtttta 3960  
 agcggtagag aaggcaaaaa acaagtagat gaacctggt ttaatctctt attgcacgaa 4020  
 acgaaattt caggagaaaa gggtttaata gggcgtaata acgtcatgtt taccctctct 4080  
 ttagcctact ttagttcagg ctattcaatc gaaacgtgcg aatataatat gtttgagttt 4140  
 aataatgat tagatcaacc cttagaagaa aaagaagtaa tcaaaattgt tagaagtgcc 4200  
 tattcagaaa actatcaagg ggctaatagg gaatacatta ccattctttg caaagcttgg 4260  
 gtatcaagtg atttaaccag taaagattta tttgccgtc aagggtggtt taaattcaag 4320  
 aaaaaagaa gcgaacgtca acgtgttcat ttgtcagaat ggaaagaaga ttaattggct 4380

tatattagcg aaaaaagcga tgtatacaag ccttatttag tgacgaccaa aaaagagatt 4440  
 agagaagtgc taggcattcc tgaacggaca ttagataaat tgctgaaggt actgaaggcg 4500  
 aatcaggaaa ttttctttaa gattaaacca ggaagaaatg gtggcattca acttgctagt 4560  
 gttaaatcat tgttgctatc gatcattaaa gtaaaaaaag aagaaaaaga aagctatata 4620  
 aaggcgctga caaattcttt tgacttagag catacattca ttcaagagac tttaacaag 4680  
 ctagcagaac gccctaaaac ggacacacaa ctcgatttgt ttagctatga tacaggctga 4740  
 aaataaaacc cgcactatgc cattacattt atatctatga tacgtgtttg tttttcttt 4800  
 gctgtttagc gaatgattag cagaaatata cagagtaaga tttaattaa ttattagggg 4860  
 gagaaggaga gagtagcccg aaaactttta gttggcttgg actgaacgaa gtgagggaaa 4920  
 ggctactaaa acgtcgaggg gcagtgagag cgaagcgaac acttgatttt ttaattttct 4980  
 atcttttata ggtcattaga gtatacttat ttgtcctata aactatttag cagcataata 5040  
 gatttattga ataggtcatt taagttgagc atattagagg aggaaaatct tggagaaata 5100  
 ttgaagaac ccgattacat ggattggatt agttcttggt gttacgtggt tttaactaa 5160  
 aagtagtgaa ttttgattt ttggtgtgtg tgtcttggtg ttagtatttg ctagtcaaag 5220  
 tgattaaata 5230

<210> 6

<211> 5906

<212> DNA

<213> Artificial

<220>

<223> Description of Artificial Sequence: plasmid pT1NX

<400> 6

gaattcgatt aagtcattt accctcttta ttagttttt cttataatct aatgataaca 60  
 tttttataat taatctataa accatatccc tctttggaat caaaatttat tatctactcc 120  
 ttttagata tgttataata caagtatcag atctgggaga ccacaacggt ttcccactag 180  
 aaataatttt gttaacttt agaaaggaga tatacgcattg aaaaaaaga ttatctcagc 240  
 tattttaatg tctacagtca tactttctgc tgcagccccg ttgtcaggtg ttacgccgg 300  
 cgacggatcc aaaagaggaa gacaataaca agcctggcaa agaagacaat aacaagcctg 360  
 gcaaagaaga caataacaag cctggcaaag aagacaaca caagcctggc aaagaagaca 420  
 acaacaagcc tggtaaagaa gacaacaaca agcctggcaa agaagacggc aacaagcctg 480  
 gtaaagaaga caacaaaaa cctggtaaag aagatggcaa caagcctggt aaagaagaca 540  
 acaaaaaacc tggtaaagaa gacggcaaca agcctggcaa agaagatggc acaaacctg 600  
 gtaaagaaga tggtaacgga gtacatgtcg ttaaacctgg tgatacagta aatgacattg 660  
 caaaagcaaa cggcactact gctgacaaaa ttgctgcaga taacaaatta gctgataaaa 720  
 acatgatcaa acctggtcaa gaactgttg ttgataagaa gcaaccagca aacctgcag 780  
 atgctaaca agctcaagca ttaccagaaa ctggcgaaga aaatccattc atcggtacaa 840  
 ctgtatttgg tggattatca ttagccttag gtgcagcgtt attagctgga cgtcgtcgcg 900  
 aactataact agtagatccg gctgctaaca aagcccgaaa ggaagctgag ttgctgctg 960  
 ccaccgtga gcaataacta gcataacccc ttggggcctc taaacgggtc ttgaggggtt 1020  
 tttgctgaa aggaggaact atatccggat gacctgcagg caagctctag aatcgatacg 1080  
 attttgaagt ggcaacagat aaaaaaagc agtttaaaat tgttgctgaa cttttaaacc 1140  
 aagcaaatac aatcattgtc gcaacagata gcgacagaga aggcgaaaac attgcctggt 1200  
 cgatcattca taaagcaaat gccttttcta aagataaaac gtataaaaga ctatggatca 1260  
 atagtttaga aaaagatgtg atccgtagcg gttttcaaaa ttgcaacca ggaatgaatt 1320  
 actatccctt ttatcaagaa gcgcaaaaga aaacgaaat gatacaccaa tcagtcaaaa 1380

aaaagatata atgggagata agacgggtcg tgttcgtgct gacttgcacc atatcataaa 1440  
 aatcgaaaca gcaaagaatg gcggaaacgt aaaagaagtt atggaaataa gacttagaag 1500  
 caaacttaag agtgtgttga tagtgcagta tcttaaaatt ttgtataata ggaattgaag 1560  
 ttaaattaga tgctaaaaat ttgtaattaa gaaggagtga ttacatgaac aaaaatataa 1620  
 aatatttca aaacttttta acgagtgaag aagtactcaa ccaaataata aaacaattga 1680  
 atttaaaaga aaccgatacc gtttacgaaa ttggaacagg taaagggcat ttaacgacga 1740  
 aactggctaa aataagtaaa caggtaacgt ctattgaatt agacagtcac ctattcaact 1800  
 tatcgtcaga aaaattaaaa ctgaatactc gtgtcacttt aattcaccaa gatattctac 1860  
 agtttcaatt ccctaacaaa cagaggtata aaattgttgg gagtattcct taccatttaa 1920  
 gcacacaaat tattaacaaa gtggtttttg aaagccatgc gtctgacatc tatctgattg 1980  
 ttgaagaagg attctacaag cgtaccttgg atattcaccg aacactaggg ttgctcttgc 2040  
 aactcaagt ctgattcag caattgctta agctgccagc ggaatgcttt catcctaaac 2100  
 caaaagtaaa cagtgtctta ataaactta cccgccatac cacagatgtt ccagataaat 2160  
 attggaagct atatacgtac tttgtttcaa aatgggtcaa tcgagaatat cgtaactgt 2220  
 ttactaaaaa tcagtttcat caagcaatga aacacgcaa agtaaacaa ttaagtaccg 2280  
 ttacttatga gcaagtattg tctatttta atagtattc attatttaac gggaggaaat 2340  
 aattctatga gtcgcttttg taaatttga aagttacacg ttactaaagg gaatgtagat 2400  
 aaattattag gtatactact gacagcttcc aaggagctaa agaggtccct agcgtctta 2460  
 tcatggggaa gctcgatca tatgcaagac aaaataaact cgcaacagca cttggagaaa 2520  
 tgggacgaat cgagaaaacc ctctttacgc tggattacat atctaataa gccgtaagga 2580  
 gacgggttca aaaagggtta aataaaggag aagcaatcaa tgcattagct agaactatat 2640  
 ttttggaca acgtggagaa ttagagaac gtgctctcca agaccagtta caaagagcta 2700  
 gtgcactaaa cataattatt aacgctataa gtgtgtggaa cactgtatat atggaaaaag 2760

ccgtagaaga attaaaagca agaggagaat ttagagaaga tttaatgcca tatgcgtggc 2820  
 cgtaggatg ggaacatata aattttcttg gagaatacaa attgaagga ttacatgaca 2880  
 ctgggcaaata gaatttacgt cctttacgta taaaagagcc gttttattct taatataacg 2940  
 gctcttttta tagaaaaaat ccttagcgtg gttttttcc gaaatgctgg cggtagccca 3000  
 agaattagaa atgagtagat caaattattc acgaatagaa tcaggaaaat cagatccaac 3060  
 cataaaaaaca ctagaacaaa ttgcaaagtt aactaactca acgctagtag tggatttaac 3120  
 cccaaatgag ccaacagaac cagagccaga aacagaatca gaacaagtaa cattggattt 3180  
 agaaatggaa gaagaaaaaa gcaatgactt cgtgtgaata atgcacgaaa tcgttgctta 3240  
 tttttttta aaagcgggtat actagatata acgaaacaac gaactgaata gaaacgaaaa 3300  
 aagagccatg acacatttat aaaatgttg acgacatttt ataatgcat agcccataa 3360  
 gattgccaaa ccaacgctta tcagttagtc agatgaactc ttccctcgta agaagttatt 3420  
 taattaactt tgtttgaaga cggtatataa ccgtactatc attatatagg gaaatcagag 3480  
 agttttcaag tatctaagct actgaattta agaattgta agcaatcaat cggaatcgt 3540  
 ttgattgctt tttttgtatt catttataga aggtggagtt tgtatgaatc atgatgaatg 3600  
 taaaacttat ataaaaata gtttattgga gataagaaaa ttagcaaata tctataact 3660  
 agaaacgttt aagaaagagt tagaaaagag aaatatctac ttagaaacaa aatcagataa 3720  
 gtatttttct tcggagggggg aagattatat atataagtt atagaaaata acaaaataat 3780  
 ttattcgatt agtggaacaaa aattgactta taaaggaaaa aaatctttt caaaacatgc 3840  
 aatattgaaa cagttgaatg aaaaagcaaa ccaagttaat taaacaacct attttatagg 3900  
 atttatagga aaggagaaca gctgaatgaa tatccctttt gttgtagaaa ctgtgcttca 3960  
 tgacggcttg ttaaagtaca aatttaaaaa tagtaaaatt cgctcaatca ctaccaagcc 4020  
 aggtaaaagc aaaggggcta tttttgcgta tcgctcaaaa tcaagcatga ttggcggtcg 4080  
 tgggtgtgtt ctgacttccg aggaagcgat tcaagaaaat caagatacat ttacacattg 4140

gacacccaac gtttatcggt atggaacgta tgcagacgaa aaccgttcat acacgaaagg 4200  
 acattctgaa aacaatttaa gacaaatcaa taccttcttt attgatttg atattcacac 4260  
 ggcaaaagaa actatttcag caagcgatat ttaacaacc gctattgatt taggttttat 4320  
 gcctactatg attatcaaat ctgataaagg ttatcaagca tttttgttt tagaaacgcc 4380  
 agtctatgtg acttcaaaat cagaatttaa atctgtcaaa gcagccaaaa taatttcgca 4440  
 aaatatccga gaatatattg gaaagcttt gccagttgat ctaacgtgta atcattttgg 4500  
 tattgctgc ataccaagaa cggacaatgt agaattttt gatcctaatt accgttattc 4560  
 ttcaaaagaa tggcaagatt ggtctttcaa acaaacagat aataagggtt ttactgttc 4620  
 aagtctaacg gttttaagcg gtacagaagg caaaaaacaa gtagatgaac cctggtttaa 4680  
 tctctattg cacgaaacga aatttcagg agaaaagggt ttaatagggc gtaataacgt 4740  
 catgtttacc ctctcttag cctacttag ttcaggctat tcaatcgaaa cgtgcgaata 4800  
 taatatgttt gagtttaata atcgattaga tcaaccctta gaagaaaaag aagtaatcaa 4860  
 aattgtaga agtgcctatt cagaaaacta tcaaggggtt aatagggaat acattaccat 4920  
 tcttgcaaa gcttgggtat caagtgttt aaccagtaaa gatttattg tccgtcaagg 4980  
 gtggtttaaa ttcaagaaaa aaagaagcga acgtcaacgt gttcattgt cagaatggaa 5040  
 agaagattta atggcttata ttagcgaaaa aagcgatgta tacaagcctt atttagtgac 5100  
 gaccaaaaa gagattagag aagtgctagg cattcctgaa cggacattag ataaattgct 5160  
 gaaggtagt aaggcgaatc aggaaattt cttaagatt aaaccaggaa gaaatggtgg 5220  
 cattcaactt gctagtgtta aatcattgtt gctatgatc attaaagtaa aaaaagaaga 5280  
 aaaagaaagc tatataaagg cgctgacaaa ttctttgac ttagagcata cattcattca 5340  
 agagacttta aacaagctag cagaacgccc taaaacggac acacaactcg attgttttag 5400  
 ctatgatata ggctgaaaat aaaacccgca ctatgccatt acatttatat ctatgatag 5460  
 tgtttgttt ttctttgctg tttagcgaat gattagcaga aatatacaga gtaagatttt 5520

aattaattat tagggggaga aggagagagt agcccgaaaa ctttagttg gcttggactg 5580  
aacgaagtga gggaaaggct actaaaacgt cgaggggcag tgagagcgaa gcgaacactt 5640  
gattttttaa tttctatct tttataggc attagagtat acttattgt cctataaact 5700  
atttagcagc ataatagatt tattgaatag gtcatttaag ttgagcatat tagaggagga 5760  
aaatcttgga gaaatatttg aagaacccga ttacatggat tggattagt cttgtggta 5820  
cgtgggtttt aactaaaagt agtgaatttt tgatttttg tgtgtgtgc ttgtgttag 5880  
tattgctag tcaaagtgat taaata 5906

<210> 7

<211> 5770

<212> DNA

<213> Artificial

<220>

<223> Description of Artificial Sequence: plasmid pT1MIL10

<400> 7

gaattcgatt aagtcattt accctcttta ttagttttt cttataatct aatgataaca 60  
ttttataat taatctataa accatatccc tctttggaat caaaatttat tatctactcc 120  
ttgtagata tgttataata caagtatcag atctgggaga ccacaacggt ttcccactag 180  
aaataatttt gttaacttt agaaaggaga tatacgcatg aaaaaaaga ttatctcagc 240  
tattttaatg tctacagtca tactttctgc tgcagcccg ttgtcaggtg ttacgcca 300  
gtacagccgg gaagacaata actgcacca ctcccagtc ggccagagcc acatgctcct 360  
agagctgcgg actgcctta gccaggtgaa gactttctt caaacaagg accagctgga 420  
caacatactg ctaaccgact ccttaatgca ggactttaag ggttacttg gttgccaagc 480

cttatcggaa atgatccagt ttacctggt agaagtgatg ccccaggcag agaagcatgg 540  
 cccagaaatc aaggagcatt tgaattccct gggtgagaag ctgaagaccc tcaggatgcg 600  
 gctgaggcgc tgtcatgat ttctcccctg tgaaaataag agcaaggcag tggagcaggt 660  
 gaagagtgat ttaataagc tccaagacca aggtgtctac aaggccatga atgaattga 720  
 catcttcac aactgcatag aagcatacat gatgatcaaa atgaaaagct aactagtaga 780  
 tccggtgct aacaaagccc gaaaggaagc tgagttggct gctgccaccg ctgagcaata 840  
 actagcataa ccccttgggg cctctaaacg ggtcttgagg ggtttttgc tgaaaggagg 900  
 aactatatcc ggatgacctg caggcaagct ctagaatcga tacgatttg aagtggcaac 960  
 agataaaaaa aagcagttta aaattgtgc tgaactttta aaacaagcaa atacaatcat 1020  
 tgtcgcaaca gatagcgaca gagaaggcga aaacattgcc tggtcgatca ttcataaagc 1080  
 aaatgccttt tctaaagata aaacgtataa aagactatgg atcaatagtt tagaaaaaga 1140  
 tgtgatccgt agcggtttc aaaattgca accaggaatg aattactatc ccttttatca 1200  
 agaagcgcaa aagaaaaacg aaatgataca ccaatcagt caaaaaaga tataatggga 1260  
 gataagacgg ttcgtgttcg tgctgacttg caccatatca taaaaatcga aacagcaaag 1320  
 aatggcggaa acgtaaaaga agttatggaa ataagactta gaagcaaact taagagtgtg 1380  
 ttgatagtgc agtatctaa aattttgtat aataggaatt gaagttaa tagatgctaa 1440  
 aaatttgtaa ttaagaagga gtgattacat gaacaaaaat ataaaatatt ctcaaaactt 1500  
 ttaacgagt gaaaaagtac tcaaccaa ataaaaacaa ttgaatttaa aagaaaccga 1560  
 taccgtttac gaaattggaa caggtaaagg gcatttaacg acgaaactgg ctaaaataag 1620  
 taaacaggta acgtctattg aattagacag tcatctattc aacttatcgt cagaaaaatt 1680  
 aaaactgaat actcgtgtca ctttaattca ccaagatatt ctacagttc aattccctaa 1740  
 caaacagagg tataaaattg ttgggagtat tccttaccat ttaagcacac aaattattaa 1800  
 aaaagtgggt ttgaaagcc atgcgtctga catctatctg attgttgaag aaggattcta 1860



caagcgtacc ttgatattc accgaacact agggttgctc ttgcacactc aagtctcgat 1920  
 tcagcaattg cttagctgc cagcggaatg ctttcacct aaacaaaag taaacagtgt 1980  
 cttataaaa cttaccgcc ataccacaga tgtccagat aaatattgga agctatatac 2040  
 gtactttgt tcaaatggg tcaatcgaga atacgtcaa ctgtttacta aaaatcagtt 2100  
 tcatcaagca atgaaacacg ccaaagtaaa caatttaagt accgttactt atgagcaagt 2160  
 attgtctatt ttaatagtt atctattatt taacgggagg aaataattct atgagtcgct 2220  
 ttgttaaatt tgaaaagtt caggttacta aagggaatgt agataaatta ttaggtatac 2280  
 tactgacagc ttccaaggag ctaaagaggt ccctagcgt cttatcatgg ggaagctcgg 2340  
 atcatatgca agacaaaata aactcgcaac agcacttgga gaaatgggac gaatcgagaa 2400  
 aacctcttt acgtggatt acatatctaa taaagccgta aggagacggg ttcaaaaagg 2460  
 tttaataaaa ggagaagcaa tcaatgcatt agctagaact atatttttg gacaacgtgg 2520  
 agaatttga gaacgtgctc tccaagacca gttacaaaga gctagtgcac taaacataat 2580  
 tattaacgt ataagtgtg ggaacactgt atataggaa aaagccgtag aagaattaaa 2640  
 agcaagagga gaatttagag aagatttaat gccatatcg tggccgtag gatgggaaca 2700  
 tatcaattt cttggagaat acaaattga aggattacat gacactgggc aaatgaattt 2760  
 acgtccttta cgtataaaag agccgttta ttctaatat aacggctctt tttatagaaa 2820  
 aaatccttag cgtggtttt ttccgaaatg ctggcggtac cccaagaatt agaaatgagt 2880  
 agatcaaatt attcacgaat agaatcagga aaatcagatc caaccataaa aacactagaa 2940  
 caaattgcaa agttaactaa ctcaacgcta gtagtggatt taatcccaa tgagccaaca 3000  
 gaaccagagc cagaaacaga atcagaacaa gtaacattgg atttagaaat ggaagaagaa 3060  
 aaaagcaatg acttcgtgtg aataatgcac gaaatcgtt cttattttt tttaaagcg 3120  
 gtatactaga tataacgaaa caacgaactg aatagaaacg aaaaagagc catgacacat 3180  
 ttataaatg ttgacgaca tttataaat gcatagcccg ataagattgc caaccaacg 3240

cttatcagtt agtcagatga actcttcct cgtaagaagt tatttaatta actttgttg 3300  
 aagacggtat ataaccgtac tatcattata tagggaaatc agagagtttt caagtatcta 3360  
 agctactgaa tttagaatt gttgaagcaat caatcggaaa tcgtttgatt gcttttttg 3420  
 tattcattta tagaagggtg agttgtatg aatcatgatg aatgtaaaac ttatataaaa 3480  
 aatagtttat tggagataag aaaattagca aatatctata cactagaaac gtttaagaaa 3540  
 gagttagaaa agagaaatat ctacttagaa acaaaatcag ataagtattt ttcttcggag 3600  
 ggggaagatt atatatataa gttaatagaa aataacaaaa taatttattc gattagtga 3660  
 aaaaaattga cttataaagg aaaaaaatct tttcaaaac atgcaatatt gaaacagttg 3720  
 aatgaaaaag caaccaagt taattaaaca acctatttta taggatttat aggaaaggag 3780  
 aacagctgaa tgaatatccc tttgttgta gaaactgtgc ttcatgacgg ctgtttaaag 3840  
 tacaattta aaaatagtaa aattcgctca atcactacca agccaggtaa aagcaaaggg 3900  
 gctatttttg cgtatcgctc aaaatcaagc atgattggcg gtcgtggtgt tgttctgact 3960  
 tccgaggaag cgattcaaga aaatcaagat acatttacac attggacacc caacgtttat 4020  
 cgttatggaa cgtatgcaga cgaaaaccgt tcatacacga aaggacattc tgaaaacaat 4080  
 ttaagacaaa tcaatacctt ctttattgat ttgatattc acacggcaaa agaaactatt 4140  
 tcagcaagcg atattttaac aaccgctatt gatttaggtt ttatgcctac tatgattatc 4200  
 aaatctgata aaggttatca agcatatttt gtttagaaa cgccagtcta tgtgacttca 4260  
 aaatcagaat ttaaactgtt caaagcagcc aaaataattt cgcaaaatat ccgagaatat 4320  
 tttgaaagt ctttgccagt tgatctaacg tgtaatcatt ttggtattgc tcgcatacca 4380  
 agaacggaca atgtagaatt tttgatcct aattaccgtt attctttcaa agaattggcaa 4440  
 gattggtctt tcaaacaac agataataag ggctttactc gttaagtct aacggtttta 4500  
 agcggtagag aaggcaaaaa acaagtagat gaacctggt ttaatctctt attgcacgaa 4560  
 acgaaatttt caggagaaaa gggtttaata gggcgtaata acgtcatgtt taccctctct 4620

ttagcctact ttagtcagg ctattcaatc gaaacgtgcg aatataatat gtttgagttt 4680  
 aataatcgat tagatcaacc cttagaagaa aaagaagtaa tcaaaattgt tagaagtgcc 4740  
 tattcagaaa actatcaagg ggctaataagg gaatacatta ccattctttg caaagcttgg 4800  
 gtatcaagtg atttaaccag taaagattta ttgtccgctc aagggtggtt taaattcaag 4860  
 aaaaaaagaa gcgaacgtca acgtgttcat ttgtcagaat ggaaagaaga tttaatggct 4920  
 tatattagcg aaaaaagcga tgtatacaag ccttatttag tgacgaccaa aaaagagatt 4980  
 agagaagtgc taggcattcc tgaacggaca ttagataaat tgctgaaggt actgaaggcg 5040  
 aatcaggaaa ttttctttaa gattaaacca ggaagaaatg gtggcattca acttgctagt 5100  
 gttaaatcat tgttgctatc gatcattaaa gtaaaaaaag aagaaaaaga aagctatata 5160  
 aaggcgctga caaattcttt tgacttagag catacattca ttcaagagac tttaacaag 5220  
 ctagcagaac gccctaaaac ggacacacaa ctcgatttgt ttagctatga tacaggctga 5280  
 aaataaaacc cgcactatgc cattacattt atatctatga tacgtgtttg tttttcttt 5340  
 gctgtttagc gaatgattag cagaaatata cagagtaaga ttttaattaa ttattagggg 5400  
 gagaaggaga gagtagcccg aaaactttta gttggcttgg actgaacgaa gtgagggaaa 5460  
 ggctactaaa acgtcgaggg gcagtgagag cgaagcgaac acttgatttt ttaattttct 5520  
 atcttttata ggtcattaga gtatacttat ttgtcctata aactatttag cagcataata 5580  
 gatttattga ataggtcatt taagtgagc atattagagg aggaaaatct tggagaaata 5640  
 ttgaagaac ccgattacat ggattggatt agttcttgtg gttacgtggt ttttaactaa 5700  
 aagtagtgaa ttttgattt ttggtgtgtg tgccttgttg ttagtatttg ctagtcaaag 5760  
 tgattaaata 5770

<210> 8

<211> 5870

<212> DNA

<213> Artificial

<220>

<223> Description of Artificial Sequence: plasmid pT1TR5AH

<400> 8

gaattcgatt aagtcattt acctctttta ttagtttttt cttataatct aatgataaca 60  
ttttataat taatctataa accatatccc tctttggaat caaaatttat tatctactcc 120  
ttttagata tgttataata caagtatcag atctgggaga ccacaacggt ttcccactag 180  
aaataatttt gtttaacttt agaaaggaga tatacgcatg aaaaaaaga ttatctcagc 240  
tattttaatg tctacagtca tactttctgc tgcagccccg ttgtcaggtg ttacgcct 300  
ggccccctct ctgggtgacc gggagaagag ggatagcttg tgtcccaag gaaagtatgt 360  
ccattctaag aacaattcca tctgctgcac caagtgccac aaaggaacct acttggtgag 420  
tgactgtccg agcccagggc gggatacagt ctgcagggag tgtgaaaagg gcacctttac 480  
ggcttcccag aattacctca ggcagtgtct cagttgcaag acatgtcgga aagaaatgtc 540  
ccaggtggag atctctcctt gccaaagtga caaggacacg gtgtgtggct gtaaggagaa 600  
ccagttccaa cgctacctga gtgagacaca ctccagtgc gtggactgca gccctgctt 660  
caacggcacc gtgacaatcc cctgtaagga gactcagaac accgtgtgta actgccatgc 720  
agggttcttt ctgagagaaa gtgagtgcgt cctttgcagc cactgcaaga aaaatgagga 780  
gtgtatgaag ttgtgcctac ctctccgct tgcaaatgtc acaaaccccc aggactcagg 840  
tactgcgat catcatcatc atcattaata gactagtaga tccggctgct acaaagccc 900  
gaaaggaagc tgagttggct gctgccaccg ctgagcaata actagcataa ccccttgggg 960  
cctctaaacg ggtcttgagg ggtttttgc tgaaaggagg aactatatcc ggatgacctg 1020  
caggcaagct ctagaatcga tacgattttg aagtggcaac agataaaaaa aagcagttta 1080  
aaattgttcg tgaactttta aaacaagcaa atacaatcat tgtcgcaaca gatagcgaca 1140

gagaaggcga aaacattgcc tggtcgatca ttcataaagc aaatgccttt tctaaagata 1200  
 aaacgtataa aagactatgg atcaatagtt tagaaaaaga tgtgatccgt agcggttttc 1260  
 aaaatttgca accaggaatg aattactatc ccttttatca agaagcgcaa aagaaaaacg 1320  
 aaatgataca ccaatcagtg caaaaaaaga tataatggga gataagacgg ttcgtgttcg 1380  
 tgctgacttg caccatatca taaaaatcga aacagcaaag aatggcggaa acgtaaaaga 1440  
 agttatggaa ataagactta gaagcaaact taagagtgtg ttgatagtc agtatcttaa 1500  
 aattttgtat aataggaatt gaagttaaatt tagatgctaa aaatttgtaa ttaagaagga 1560  
 gtgattacat gaacaaaaat ataaaatatt ctcaaaactt ttaacgagt gaaaaagtac 1620  
 tcaaccaaatt aataaaacaa ttgaatttaa aagaaaccga taccgtttac gaaattggaa 1680  
 caggtaaagg gcatttaacg acgaaactgg ctaaaataag taaacaggta acgtctattg 1740  
 aattagacag tcattctattc aacttatcgt cagaaaaatt aaaactgaat actcgtgtca 1800  
 cttaattca ccaagatatt ctacagtttc aattccctaa caaacagagg tataaaattg 1860  
 ttgggagtat tccttaccat ttaagcacac aaattattaa aaaagtgggt ttgaaagcc 1920  
 atgcgtctga catctatctg attgttgaag aaggattcta caagcgtacc ttggatattc 1980  
 accgaacact aggggttgctc ttgcacactc aagtctcgat tcagcaattg cttaaagtgc 2040  
 cagcggaatg ctttcatcct aaaccaaag taaacagtgt cttataaaaa cttacccgcc 2100  
 ataccacaga tgttcagat aaatattgga agctatatac gtactttgtt tcaaaatggg 2160  
 tcaatcgaga atatcgtaa ctgtttacta aaaatcagtt tcatcaagca atgaaacacg 2220  
 ccaaagtaaa caatttaagt accgttactt atgagcaagt attgtctatt ttaatatgtt 2280  
 atctattatt taacgggagg aaataattct atgagtcgct ttgttaaatt tggaaagtta 2340  
 cagtttacta aagggaatgt agataaatta ttaggtatac tactgacagc ttccaaggag 2400  
 ctaaagaggt ccctagcgct cttatcatgg ggaagctcgg aicatatgca agacaaaata 2460  
 aactcgcaac agcacttgga gaaatgggac gaatcgagaa aaccctcttt acgctggatt 2520

acatatctaa taaagccgta aggagacggg ttcaaaaagg tttaaataaa ggagaagcaa 2580  
 tcaatgcatt agctagaact atatTTTTg gacaacgtgg agaatttaga gaacgtgctc 2640  
 tccaagacca gttacaaaga gctagtgcac taaacataat tattaacgct ataagtgtgt 2700  
 ggaacactgt atatatggaa aaagccgtag aagaattaaa agcaagagga gaatttagag 2760  
 aagatttaat gccatatgcg tggccgttag gatgggaaca tatcaatttt ctgggagaat 2820  
 acaaatttga aggattacat gacactgggc aaatgaattt acgtccttta cgtataaaag 2880  
 agccgtttta ttcttaatat aacggctctt tttagataaa aaatccttag cgtgggtttt 2940  
 ttccgaaatg ctggcggtag cccaagaatt agaaatgagt agatcaaatt attcacgaat 3000  
 agaatcagga aaatcagatc caaccataaa aacactagaa caaattgcaa agttaactaa 3060  
 ctcaacgcta gtagtggatt taatcccaaa tgagccaaca gaaccagagc cagaaacaga 3120  
 atcagaacaa gtaacattgg atttagaaat ggaagaagaa aaaagcaatg acttcgtgtg 3180  
 aataatgcac gaaatcgtg cttatttttt tttaaagcg gtatactaga tataacgaaa 3240  
 caacgaactg aatagaaacg aaaaaagagc catgacacat ttataaatg ttgacgaca 3300  
 tttataaat gcatagcccg ataagattgc caaccaacg cttatcagtt agtcagatga 3360  
 actcttcct cgtagaagt tatttaatta actttgttg aagacggtat ataaccgtac 3420  
 tatcattata tagggaaatc agagagtttt caagtatcta agctactgaa tttaagaatt 3480  
 gttaagcaat caatcgaaa tcgttgatt gcttttttg tattcattta tagaaggtgg 3540  
 agttgtatg aatcatgatg aatgtaaaac ttatataaaa aatagtttat tggagataag 3600  
 aaaattagca aatatctata cactagaac gttaagaaa gagttagaaa agagaaatat 3660  
 ctacttagaa acaaaatcag ataagtattt ttctcggag ggggaagatt atatatataa 3720  
 gttaatagaa aataacaaaa taatttattc gattagtga aaaaaattga cttataaagg 3780  
 aaaaaaatct tttcaaac atgcaatatt gaaacagtg aatgaaaaag caaccaagt 3840  
 taattaaaca acctatttta taggatttat aggaaaggag aacagctgaa tgaatatccc 3900

tttgttgta gaaactgtgc ttcatgacgg cttgttaaag tacaaattta aaaatagtaa 3960  
 aattcgctca atcactacca agccaggtaa aagcaaaggg gctatttttg cgtatcgctc 4020  
 aaaatcaagc atgattggcg gtcgtggtgt tgttctgact tccgaggaag cgattcaaga 4080  
 aatcaagat acatttacac attggacacc caacgtttat cgttatggaa cgatgcaga 4140  
 cgaaaaccgt tcatacacga aaggacattc tgaaaacaat ttaagacaaa tcaatacctt 4200  
 ctttattgat ttgatattc acacggcaaa agaaactatt tcagcaagcg atattttaac 4260  
 aaccgctatt gatttaggtt ttatgcctac tatgattatc aaatctgata aaggttatca 4320  
 agcatatfff gtttagaaa cgccagtcta tgtgacttca aaatcagaat taaatctgt 4380  
 caaagcagcc aaaataattt cgcaaaatat ccgagaatat ttggaaagt cttgccagt 4440  
 tgatctaagc tgaatcatt ttgtattgc tcgcatacca agaacggaca atgtagaatt 4500  
 tttgatcct aattaccgtt attctttcaa agaattggcaa gattggtctt tcaacaaac 4560  
 agataataag ggctttactc gttcaagtct aacggtttta agcgggtacag aaggcaaaaa 4620  
 acaagtagat gaaccctggt ttaatctctt attgcacgaa acgaaattt caggagaaaa 4680  
 gggtttaata gggcgtaata acgtcatgtt taccctctct ttagcctact ttagttcagg 4740  
 ctattcaatc gaaacgtgcg aatataatat gtttgagttt aataatcgat tagatcaacc 4800  
 cttagaagaa aaagaagtaa tcaaaattgt tagaagtgcc tattcagaaa actatcaagg 4860  
 ggctaataagg gaatacatta ccattctttg caaagcttgg gtatcaagtg atttaaccag 4920  
 taaagattta ttgtccgctc aagggtggtt taaattcaag aaaaaagaa gcgaacgtca 4980  
 acgtgttcat ttgtcagaat ggaaagaaga ttaattggct tatattagcg aaaaaagcga 5040  
 tgtatacaag ccttathtag tgacgaccaa aaaagagatt agagaagtgc taggcattcc 5100  
 tgaacggaca ttagataaat tgctgaaggt actgaaggcg aatcaggaaa tttcttta 5160  
 gattaaacca ggaagaaatg gtggcattca acttgctagt gttaaatcat tgttgctatc 5220  
 gatcattaa gtaaaaaaag aagaaaaaga aagctatata aaggcgctga caaattcttt 5280

tgacttagag catacattca ttcaagagac tttaaacaag ctagcagaac gccctaaaac 5340  
 ggacacacaa ctcgatttgt ttagctatga tacaggctga aaataaaacc cgcactatgc 5400  
 cattacattt atatctatga tacgtgttg tttttcttt gctgtttagc gaatgattag 5460  
 cagaaatata cagagtaaga tttaattaa ttattagggg gagaaggaga gagtagcccg 5520  
 aaaactttta gttggcttgg actgaacgaa gtgagggaaa ggctactaaa acgtcgaggg 5580  
 gcagtgagag cgaagcgaac acttgatttt ttaattttct atcttttata ggtcattaga 5640  
 gtatacttat ttgtcctata aactatttag cagcataata gatttattga ataggtcatt 5700  
 taagttgagc atattagagg aggaaaatct tggagaaata ttgaagaac ccgattacat 5760  
 ggattggatt agttcttgtg gttacgtggt tttaactaa aagtagtgaa ttttgattt 5820  
 ttggtgtgtg tgtcttgtg ttagtatttg ctagtcaaag tgattaaata 5870



# **APPENDIX D**

**(Article, *Science* 2000, p. 1352-55 )**

**(Serial No. 09/838,718)**

idence for such stabilization has been seen in solution studies, and direct MHC class II-CD4 binding was not detected (31). These results and the data presented here instead suggest that the function of CD4 may be to "boost" or "trigger" the early phase of activation. Once that process is under way, CD4 seems to be excluded from the central core of the synapse, perhaps owing to the formation of some lattice-like structure by the remaining molecules.

## References and Notes

1. A. Weiss and D. R. Littman, *Cell* 76, 263 (1994).
2. R. König, L. Huang, R. N. Germain, *Nature* 356, 796 (1992).
3. C. Cammarota et al., *Nature* 356, 799 (1992).
4. A. Kupfer and S. J. Singer, *J. Exp. Med.* 170, 1697 (1989).
5. P. Marack et al., *J. Exp. Med.* 158, 1077 (1983).
6. J. Hampl, Y.-H. Chein, M. M. Davis, *Immunity* 7, 379 (1997).
7. J. Madrenas, L. A. Chau, J. Smith, J. A. Bluestone, R. N. Germain, *J. Exp. Med.* 185, 219 (1997).
8. J. M. Turner et al., *Cell* 60, 753 (1990).
9. D. B. Straus and A. Weiss, *Cell* 70, 585 (1992).
10. M. M. Davis et al., *Annu. Rev. Immunol.* 16, 523 (1998).
11. C. A. Janeway Jr., *Annu. Rev. Immunol.* 10, 645 (1992).
12. C. R. F. Monks, B. A. Freiberg, H. Kupfer, N. Siatky, A. Kupfer, *Nature* 395, 82 (1998).
13. A. Grakoui et al., *Science* 285, 221 (1999).
14. C. Wülfing, M. D. Sjaastad, M. M. Davis, *Proc. Natl. Acad. Sci. U.S.A.* 95, 8302 (1998).
15. J. Kaye, S. Porcelli, J. Tite, B. Jones, C. A. Janeway Jr., *J. Exp. Med.* 158, 836 (1983).
16. GFP fusion constructs were transfected into D10 by electroporation as described (32). After 1 to 2 weeks, these cultures were expanded into 24-well dishes, screened by flow cytometry, and sorted for GFP expression. Established clones were subjected to phenotypic analysis by staining for CD4, CD3ε, TCR, CD28, and CD25 as well as proliferation analysis against CA 134-147 and the variant peptides EBT and ISG.
17. Supplemental Web material is available at Science On-line at [www.sciencemag.org/feature/data/1050535.shl](http://www.sciencemag.org/feature/data/1050535.shl).
18. Clones were maintained by weekly restimulations as described (15), except that cultures were typically not supplemented with exogenous interleukin-2. Activation experiments were performed from 5 to 10 days after the stimulation culture. CH27 is a B cell lymphoma that expresses IA<sup>b</sup>, B7-1, and B7-2. Experiments were done at 37°C as described (14).
19. GFP intensity data were corrected both for background intensity and for photobleaching. Background level was measured by imaging a dish in the absence of cells. This background level was subtracted from intensity data to obtain background-subtracted data sets. For bleaching, a nonreactive cell was analyzed for surface intensity over time, and the intensity over time was fitted to an exponential decay curve ( $I_t = I_0 10^{-kt}$ , where  $k$  is the decay constant,  $t$  is the number of times the fluorophore was illuminated at a constant rate, and  $I_t$  and  $I_0$  are the initial intensities and intensities at time  $t$ ). The decay constant  $k$  was used to calculate a correction factor ( $1/10^{kt}$ ) for each time point in the experimental data sets. Typical decay constants were ~0.01, giving half-lives of about 30 illuminations. Typical experiments involved about 60 to 80 illuminations.
20. Intensities from 340- and 380-nm excitations of Fura-2 were used to make a ratio image. Background calcium levels were obtained from at least five frames before activation and were similar to nonreactive cells from other portions of the field. Increases of 30% above background were found to be well above random fluctuations, and the onset of agonist-driven reactions typically was characterized by at least a 100% increase in a single 15-s time period. Individual cells were analyzed for maximal pixel intensities along the leading edge of cells by a line-scan function. All collected  $x$  planes were analyzed and compared to the average from three intensity line scans (taken at different  $x$  planes) around the circumference of the cell of interest. Background- and bleach-corrected data were used for all analyses. Rectangular  $x$ - $y$  regions encompassing the estimated interface were determined from the differential interference contrast (DIC) image, and  $z$  stacks encompassing these regions were used for the interface projections. Max intensity projections were used (Metamorph, Universal Imaging, PA).
21. C. Wülfing and M. M. Davis, unpublished observations.
22. ———, *Science* 282, 2266 (1998).
23. A. Viola, S. Schroeder, Y. Sakakibara, A. Lanzavecchia, *Science* 283, 680 (1999).
24. S. Caplan, S. Ziegler, L. Wang, M. Boniash, *Proc. Natl. Acad. Sci. U.S.A.* 92, 4768 (1995).
25. M. M. Roedizal, B. Malissen, T. H. Finkel, *Immunity* 3, 623 (1995).
26. J. R. Cochran, T. O. Cameron, L. J. Stem, *Immunity* 12, 241 (2000).
27. J. J. Boniface et al., *Immunity* 9, 459 (1998).
28. M. D. Bootman, M. J. Berridge, P. Lipp, *Cell* 91, 367 (1998).
29. Z. Reich et al., *Nature* 387, 617 (1997).
30. P. A. Negulescu, N. Shastri, M. D. Calahan, *Proc. Natl. Acad. Sci. U.S.A.* 91, 2873 (1994).
31. N. Barclay and P. A. van der Merwe, unpublished observations.
32. J. Kaye and S. M. Hedrick, *Nature* 336, 580 (1988).
33. A. Viola and A. Lanzavecchia, *Science* 273, 104 (1996).
34. M. F. Krummel, M. D. Sjaastad, C. Wülfing, M. M. Davis, data not shown.
35. We are grateful to J. Huppa, C. Sumen, and L. Richie for critical comments and advice. We thank C. Terhorst and J. Parnes for supplying the cDNAs for CD3ε and CD4, respectively. We also thank the Stanford Shared FACS Facility for assistance in cell sorting. Supported by a postdoctoral award from the National Institutes of Health (M.F.K.) and by the Howard Hughes Medical Institute and NIH.

20 March 2000; accepted 13 July 2000

## Treatment of Murine Colitis by *Lactococcus lactis* Secreting Interleukin-10

Lothar Steidler,<sup>1\*†</sup> Wolfgang Hans,<sup>1†</sup> Lieven Schotte,<sup>1</sup> Sabine Neirynck,<sup>1</sup> Florian Obermeier,<sup>2</sup> Werner Falk,<sup>2</sup> Walter Fiers,<sup>1</sup> Erik Remaut<sup>1</sup>

The cytokine interleukin-10 (IL-10) has shown promise in clinical trials for treatment of inflammatory bowel disease (IBD). Using two mouse models, we show that the therapeutic dose of IL-10 can be reduced by localized delivery of a bacterium genetically engineered to secrete the cytokine. Intragastric administration of IL-10-secreting *Lactococcus lactis* caused a 50% reduction in colitis in mice treated with dextran sulfate sodium and prevented the onset of colitis in IL-10<sup>-/-</sup> mice. This approach may lead to better methods for cost-effective and long-term management of IBD in humans.

Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, is a significant public health problem in Western societies, affecting 1 in 1000 individuals, yet its etiology remains poorly understood. IBD is characterized clinically by chronic inflammation in the large and/or small intestine, the symptoms of which include diarrhea, abdominal pain, weight loss, and nausea. Death can result, in extreme cases, from malnutrition, dehydration, and anemia. IBD is thought to arise from interacting genetic and environmental factors (1) and may involve abnormal T cell responses to commensal microflora (2-4). Biologically based therapies such as antibodies to tumor necrosis factor (TNF), which is a strong

proinflammatory mediator (5-7), and recombinant IL-10 (8) can ameliorate the disorder.

Because IL-10 has a central role in down-regulating inflammatory cascades (9) and matrix metalloproteinases (10), it is a likely candidate for use in therapeutic intervention. In this study we have tested a new method of delivering IL-10: in situ synthesis by genetically engineered bacteria (*Lactococcus lactis*), in two mouse models of the disease, one involving treatment of chronic colitis induced by 5% dextran sulfate sodium (DSS) (11) and one involving prevention of colitis that spontaneously develops in IL-10<sup>-/-</sup> mice (12). We show that this approach, which depends on in vivo synthesis of IL-10, requires much lower doses than systemic treatment. Neither mouse model mimics all aspects of human IBD, but such models are essential for development of new therapeutic approaches to IBD (13-20).

*L. lactis* is a nonpathogenic, noninvasive, noncolonizing Gram-positive bacterium, mainly used to produce fermented foods. We previously constructed recombinant *L. lactis* strains for production and in vivo delivery of cytokines

<sup>1</sup>Department of Molecular Biology, Ghent University and Flanders Interuniversity Institute for Biotechnology, K. L. Ledeganckstraat 35, 9000 Ghent, Belgium.

<sup>2</sup>Department of Internal Medicine I, University of Regensburg, Franz-Josef-Strauss-Allee 11, 93042 Regensburg, Germany.

\*To whom correspondence should be addressed. E-mail: [lothar.steidler@mbb.rug.ac.be](mailto:lothar.steidler@mbb.rug.ac.be)

†These authors contributed equally to this work.

(21–23). We have now engineered an *L. lactis* strain (*LL-mIL10*) for secretion of biologically active murine IL-10 (mIL-10) (Fig. 1) (24, 25).

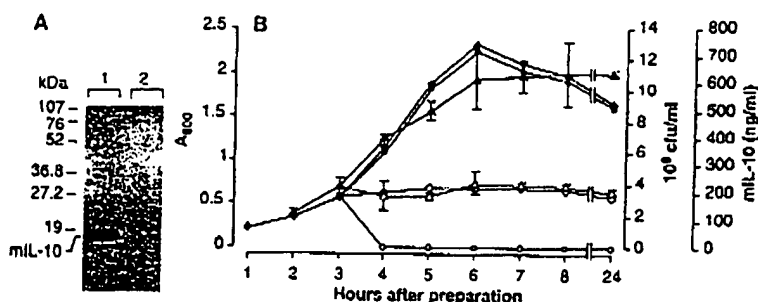
To evaluate the efficacy of the new therapeutic concept, we applied daily intragastric inocula (24) of  $2 \times 10^7$  *LL-mIL10* or control *L. lactis* (25) to mice in which chronic colitis had been induced by four cycles of administration of DSS in the drinking water for 7 days, alternating with 10-day periods of recovery (11, 26). Treatment was arbitrarily initiated at day 21 after the fourth DSS administration. The predominant epithelial damage was loss of goblet cells and crypts (Fig. 2A) (25). A lymphocytic infiltrate was largely restricted to the thickened mucosa of the middle and distal colon; submucosa was affected in about 10% of mice. Inflammation typically persisted for at

least 3 months. The chronic phase of inflammation showed complete regeneration of the intestinal epithelial lining. The range of inflammation is shown in histological images from the distal colon (Fig. 2A) and middle colon (25).

Histological scores were devised to allow quantification of histological changes (Fig. 3A). Scores from individual mice ( $n = 10$ ) after treatment with different *L. lactis* strains were recorded after blinded interpretation of sections from the distal colon (26). Untreated healthy mice had a histological score of 1, whereas mice with the induced chronic colitis and mock-treated control mice had a score of  $\sim 5$ . Mice treated for 14 days with *LL-mIL10* given by gastric catheter, followed by 14 days of recovery, had an average histological score of  $\sim 3$ . This represents a nearly 50% decrease

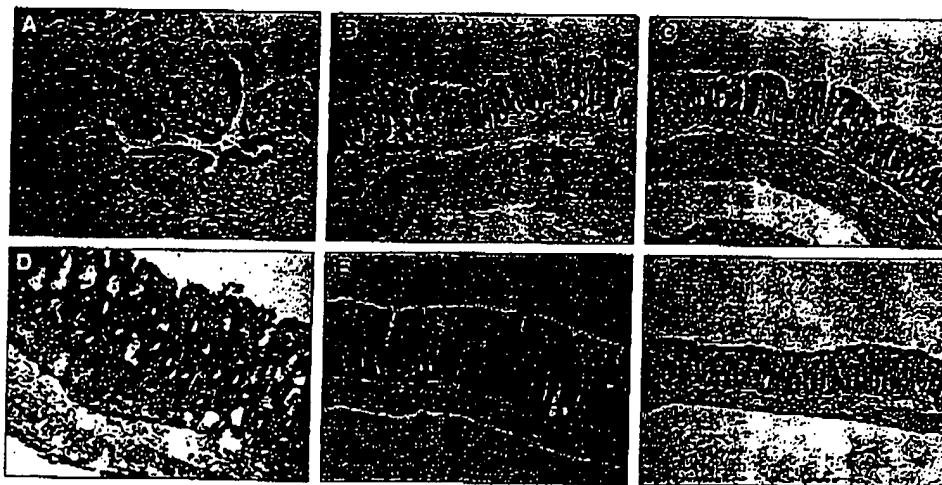
0.0151) in pathological symptoms, a more pronounced improvement than that obtained with standard systemic treatment with TNF monoclonal antibodies (mAbs), which leads to a 30 to 40% decrease in inflammation (26). In the *LL-mIL10*-treated group, colons of 4 of 10 mice had the histological characteristics of healthy mice, and 4 showed minor patchy remnants of inflammation (25); in 2 mice, larger areas remained affected. No further improvement was observed when *LL-mIL10* treatment was extended to 4 weeks; when the daily inoculum was  $10^9$  bacteria rather than  $2 \times 10^7$ , the improvement in pathology was less pronounced (27). Mice with DSS-induced colitis occasionally developed adenomas (25), corresponding to development in humans of adenocarcinoma often seen associated with ulcerative colitis. By contrast, no adenomas were seen in mice treated for 14 days with *LL-mIL10*.

We also evaluated the ability of *LL-mIL10* to prevent the onset of colitis in *IL-10*<sup>-/-</sup> mice, which spontaneously develop colitis at age 3 to 8 weeks (12). This progressive disease is characterized by multifocal inflammatory cell infiltrates (mononuclear cells and neutrophils), moderate epithelial hyperplasia, and slight mucin depletion from goblet cells. Other work has shown that treatment of 3-week-old *IL-10*<sup>-/-</sup> mice with recombinant mIL-10, interferon- $\gamma$  mAbs, or IL-12 mAbs prevents the onset of colitis (16, 28). In our studies, the untreated *IL-10*<sup>-/-</sup> mice showed less severe inflammation than that observed by other workers (16, 29). We treated 3-week-old mice ( $n = 5$ ) by daily intragastric inocula of  $2 \times 10^7$  or  $10^9$  *LL-mIL10* or control *L. lactis* as for the DSS-induced colitis model (Fig. 2) (25). Untreated mice had a mean histological score of  $\sim 4.5$ , whereas the *LL-mIL10*-treated group had a mean score of 1.5, which only slightly exceeds values reported for control mice (16) (Fig. 3B). In contrast to results with DSS-induced colitis, treatment effi-



**Fig. 1.** mIL-10 synthesis by *LL-mIL10*. (A) Western blot analysis of culture supernatant proteins from the mIL-10 producer strain *LL-mIL10* (lane 1) and the vector control *LL-TREX1* (lane 2) (24, 25), revealed with anti-mIL-10 (Pepro Tech EC, London, UK). The position of mIL-10 is indicated. The concentration of mIL-10 in the culture supernatant was 3  $\mu$ g/ml, as determined by enzyme-linked immunosorbent assay. The biological activity in the culture supernatant was estimated at 10,000 U/ml in a cell proliferation bioassay with the IL-10-dependent mast cell line MC/9 (33). When compared with a standard of known activity (BioSource International, Camarillo, California), the recombinant mIL-10 from the *LL-mIL10* culture supernatant revealed full specific biological activity. The NH<sub>2</sub>-terminus of this protein was determined, by automated Edman degradation, to be Gln-Tyr-Ser-Arg-Glu, which is identical to that of native mIL-10. (B)  $A_{600}$  ( $\bullet$ ), colony-forming units (CFU) ( $\bullet$ ), and mIL-10 concentration ( $\Delta$ ) for an *LL-mIL10* culture, prepared as for the inoculation of mice with  $2 \times 10^7$  bacteria (24). Open symbols are for corresponding profiles of an identical culture that was UV-irradiated 3 hours after preparation. UV-irradiation immediately blocked the accumulation of mIL-10 in the culture supernatant and reduced the CFU count by 6 logarithmic units. No lysis was observed (25).

**Fig. 2.** Intestinal histology of murine colitis models. Images represent sections of the distal colon (magnification,  $\times 100$ ). Hematoxylin and eosin staining. (A to C) Inflammation in the DSS-induced colitis model, established in female Balb/c mice. Lymphocytic infiltrate and disturbance of tissue architecture was observed in untreated mice (A). After treatment by 14 daily intragastric inocula of  $2 \times 10^7$  *LL-mIL10* per mouse followed by 14 days of recovery, the lymphocytic infiltrate was reduced and the tissue architecture was restored (B). Healthy control mice (C). (D to F) Images from 7-week-old female 129Sv/Ev *IL-10*<sup>-/-</sup> mice. Lymphocytic infiltrate is apparent in untreated mice (D) and mice treated for 4 weeks by daily intragastric inocula of  $2 \times 10^7$  *LL-TREX1* (E) but not in mice treated for 4 weeks by daily intragastric inocula of  $2 \times 10^7$  *LL-mIL10* (F).



cacy was the same for both inocula concentrations (27).

We confirmed that the therapeutic effect was due to mIL-10 synthesized *de novo* by *LL-mIL10* rather than to residual amounts of mIL-10 in the inocula. Indeed, because of the culture conditions used, a minor amount of mIL-10 (40 ng) (Fig. 1B) was present in the supernatant of the inoculum. The fate of this residual mIL-10 is likely acid denaturation, followed by breakdown in the stomach and duodenum (25). Diseased mice (DSS-induced colitis) treated for 2 or 4 weeks with ultraviolet (UV)-killed *LL-mIL10* cultures (Fig. 1B) (25) showed no difference in colon histology compared with control mice positive for colitis (Fig. 3A). This result indicates that the therapeutic effects require physiologically active *LL-mIL10*.

We also investigated the synthesis of recombinant mIL-10 by *LL-mIL10* in the intestine of IL-10<sup>-/-</sup> mice, which cannot themselves synthesize mIL-10. After administration of a total of  $2.4 \times 10^{10}$  *LL-mIL10* (as serial inocula), we detected  $7 \times 10^4$  *LL-mIL10* and 7 ng of mIL-10 in the colon (25). Hence, these bacteria can actively produce mIL-10 in the colon, albeit at a lower yield than that observed in culture. This result agrees well with recent findings that *L. lactis* is metabolically active in all compartments of the intestinal tract (30). Although *LL-mIL10* organisms were present in other areas of the gastrointestinal tract (cecum, ileum, jejunum, and stomach), mIL-10 was not detectable there. Perhaps mIL-10 reached detectable levels only in the colon because in

this part of the intestine the protein is not degraded, and the contents move slowly enough to allow its accumulation.

We compared the performance of *LL-mIL10*-mediated mIL-10 delivery with that of standard anti-inflammatory methods: systemic treatment (five daily intraperitoneal injections) with recombinant mIL-10, IL-12 mAbs (31), or dexamethasone. All therapies decreased inflammation in DSS-induced colitis by ~50% (Fig. 3A). Our method, however, required a much lower amount of mIL-10. We estimated that 14 daily inoculations of  $2 \times 10^7$  *LL-mIL10* delivered ~1 U of mIL-10 per mouse (25), i.e., an amount that is several orders of magnitudes lower than the optimized total amount of intraperitoneally injected mIL-10 ( $1.25 \times 10^4$  U per mouse).

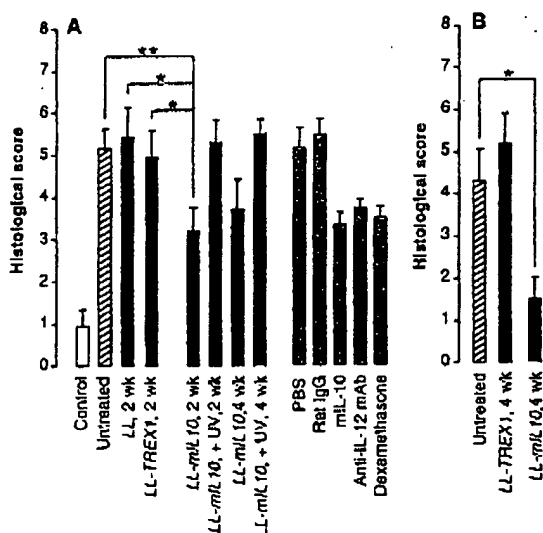
We propose two possible routes by which mIL-10 might reach its therapeutic target. The lactococci may produce mIL-10 in the lumen, and the protein may diffuse to responsive cells in the epithelium or the lamina propria. Alternatively, the lactococci may be taken up by M cells (bacterial size and shape would allow this), and the major part of the effect may be due to recombinant mIL-10 production *in situ* in intestinal lymphoid tissue. Both routes may involve paracellular transport mechanisms that are enhanced in inflammation. After transport, mIL-10 may directly down-regulate inflammation. Alternatively, autocrine mIL-10 secretion by lymphoid cells, as shown by transfer of Tr1 cells (32), epithelial cells, or both, may be induced and may enhance repair.

In summary, the method described here—cost-effective localized delivery of a therapeutic agent that is actively synthesized *in situ* by food-grade bacteria—may have potential clinical applications for treatment of IBD, particularly as an alternative to systemic treatment. In principle, the method may also be useful for intestinal delivery of other protein therapeutics that are unstable or difficult to produce in large quantities.

#### References and Notes

1. R. B. Sartor, *Am. J. Gastroenterol.* 92, 55 (1997).
2. H. C. Rath et al., *J. Clin. Invest.* 98, 945 (1996).
3. R. K. Sellon et al., *Infect. Immun.* 66, 5224 (1998).
4. T. T. MacDonald, G. Monteleone, S. L. Pender, *Scand. J. Immunol.* 51, 2 (2000).
5. S. R. Targan et al., *N. Engl. J. Med.* 337, 1029 (1997).
6. H. M. van Dullemen et al., *Gastroenterology* 109, 129 (1995).
7. G. D'Haens et al., *Gastroenterology* 116, 1029 (1999).
8. S. J. van Deventer, C. O. Elson, R. N. Fedorak, *Gastroenterology* 113, 383 (1997).
9. P. Stordeur and M. Goldman, *Int. Rev. Immunol.* 16, 501 (1998).
10. S. L. Pender et al., *Gastroenterology* 115, 573 (1998).
11. I. Okayasu et al., *Gastroenterology* 98, 694 (1990).
12. R. Kuhn, J. Lohler, D. Rennick, K. Rajewsky, W. Müller, *Cell* 75, 263 (1993).
13. M. F. Neurath, I. J. Fuss, B. L. Kelsall, E. Stuber, W. Strober, *J. Exp. Med.* 182, 1281 (1995).
14. R. O. Ehrhardt, B. R. Ludviksson, B. Gray, M. F. Neurath, W. Strober, *J. Immunol.* 158, 566 (1997).
15. M. Boirivant, I. J. Fuss, A. Chu, W. Strober, *J. Exp. Med.* 188, 1929 (1998).
16. D. J. Berg et al., *J. Clin. Invest.* 98, 1010 (1996).
17. D. Picarella et al., *J. Immunol.* 158, 2099 (1997).
18. D. K. Podolsky et al., *J. Clin. Invest.* 92, 372 (1993).
19. K. L. Madsen, J. S. Doyle, L. D. Jewell, M. M. Tavennini, R. N. Fedorak, *Gastroenterology* 116, 1107 (1999).
20. J. K. Collins et al., *Gastroenterology* 116, G2981 (1999).
21. L. Steidler et al., *Appl. Environ. Microbiol.* 61, 1627 (1995).
22. L. Steidler, W. Fiers, E. Remaut, in *Current Advances in Genetics, Metabolism and Application of Lactic Acid Bacteria*, F. Botoğlu and B. Ray, Eds. (NATO ASI Series H, Springer, Berlin, 1996), vol. 98, pp. 63–79.
23. L. Steidler et al., *Infect. Immun.* 66, 3183 (1998).
24. Standard media were M17 (Difco Laboratories, Detroit, MI) and M9 [6 g of Na<sub>2</sub>HPO<sub>4</sub>, 3 g of KH<sub>2</sub>PO<sub>4</sub>, 1 g of NH<sub>4</sub>Cl, 0.5 g of NaCl, 0.12 g of MgSO<sub>4</sub>, 0.01 g of CaCl<sub>2</sub>, and 5 g of Casitone (Difco Laboratories) per liter], or GM17 and GM9 when supplemented with 0.5% glucose. BM9 means GM9 buffered with 50 mM CO<sub>2</sub>. During growth in GM17 or GM9, *L. lactis* produces lactate, so that the acidity of the culture drops to pH 5.5. Probably because of its known acid sensitivity, no recombinant mIL-10 was detected in the *LL-mIL10* culture supernatants. We tested a series of CO<sub>2</sub>-buffered BM9 cultures, with the pH ranging from 7 to 9.5. For optimal mIL-10 protein synthesis, saturated cultures that had grown overnight in GM17 were diluted 1:100 in fresh GM17 and grown for 3 hours. Bacteria were collected by centrifugation, were resuspended in an equal volume of BM9 buffered to pH 8.5, and were grown for 5 hours more, at which point the pH was ~7.0. For all intragastric inoculations, BM9 was used at an initial pH of 8.5. Stock suspensions of all strains were stored at -20°C in 50% glycerol-GM17. Precultures were grown daily by diluting stock suspensions 200-fold in fresh GM17. These cultures grow to saturation within 16 hours when incubated at 30°C. Bacterial cells either were collected at this stage and concentrated 10-fold in BM9 medium (given as a 100-μl inoculum of 10<sup>9</sup> bacteria) or were diluted 25-fold in BM9 and grown for 3 hours at 30°C (given as a 200-μl inoculum of 2 × 10<sup>7</sup> bacteria). Earlier studies had shown that bacteria from both types of suspensions secrete similar quantities of recombinant proteins.

Fig. 3. Statistical evaluation of colon histology. Colon sections were randomly numbered and interpreted in a blinded manner. Scores from individual mice were subsequently decoded, and re-grouped numbers were analyzed statistically. Bars represent the mean  $\pm$  SEM. \* $P < 0.025$ ; \*\* $P = 0.0151$ . (A) Histological scores (sum of epithelial damage and lymphoid infiltrate, both ranging from 0 to 4) for the distal colon of groups ( $n = 10$ ) of control female Balb/c mice (white bar) and of female Balb/c mice with DSS-induced colitis that were untreated (hatched bar), treated with the indicated *L. lactis* cultures (black bars), or treated with five daily intraperitoneal injections of the compounds indicated (gray bars) (mIL-10: 5  $\mu$ g per mouse per day; anti-IL-12: 1 mg per mouse per day; dexamethasone: 5  $\mu$ g per mouse per day; rat IgG: 5  $\mu$ g per mouse per day). Mice treated daily for 2 or 4 weeks (wk) with  $2 \times 10^7$  mIL-10-producing *LL-mIL10* showed significantly reduced inflammation when compared with untreated or control-treated (*LL* or *LL-TREX1*) mice. This effect was not observed when *LL-mIL10* cultures were UV-killed (+UV). (B) Histological scores (sum of the degrees of inflammation in the proximal, middle, and distal colon, all ranging between 0 and 4) obtained after blinded interpretation of groups ( $n = 5$ ) of 7-week-old untreated (hatched bar), *LL-TREX1*-treated, or *LL-mIL10*-treated female 129 Sv/Ev IL-10<sup>-/-</sup> mice (black bars). *LL-mIL10*-treated mice showed significantly less inflammation than untreated mice.



25. For further details, see Science Online at [www.sciencemag.org/feature/data/1047997.shl](http://www.sciencemag.org/feature/data/1047997.shl).
26. C. Kojouharoff et al., *Clin. Exp. Immunol.* 107, 353 (1997).
27. L. Steidler et al., data not shown.
28. D. M. Rennick, M. M. Fort, N. J. Davidson, *J. Leukocyte Biol.* 61, 389 (1997).
29. Mice were housed in ventilated cages in which incoming and outgoing air was filtered over a high-efficiency particulate air filter. All manipulations were performed inside a class II biosafety hood. This

"clean" housing may account for the lower degree of intestinal inflammation in our mice than that previously reported in the literature.

30. G. Corthier, C. Delorme, S. D. Ehrlich, P. Renault, *Appl. Environ. Microbiol.* 64, 2721 (1998).
31. M. Wysocki et al., *Eur. J. Immunol.* 25, 672 (1995).
32. H. Groux et al., *Nature* 389, 737 (1997).
33. L. Thompson-Snipes et al., *J. Exp. Med.* 173, 507 (1991).
34. We thank I. Bruggeman, H. Devlies, K. Pollinger, and K. Van Laer for technical assistance; J. Wells for pTREX1; T.

Velu for mIL-10 cDNA; K. Madsen for 129SvEv IL-10<sup>-/-</sup> mice; G. Trinchieri for anti-IL-12-producing C17.8 hybridoma cells; J. Van Snick for MC/9 cells; M. Proet for automated tissue processing; and C. Cuvelier, K. Madsen, and P. Vandenabeele for helpful discussion and critically reading the manuscript. L.S. is a fellow with the Vlaams Instituut voor de Bevordering van het Wetenschappelijk-technologisch Onderzoek in de Industrie. Supported by grants 1.5567.98N and G005097 of the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen.

16 December 1999; accepted 10 July 2000

## Whistle Matching in Wild Bottlenose Dolphins (*Tursiops truncatus*)

Vincent M. Janik

Dolphin communication is suspected to be complex, on the basis of their call repertoires, cognitive abilities, and ability to modify signals through vocal learning. Because of the difficulties involved in observing and recording individual cetaceans, very little is known about how they use their calls. This report shows that wild, unrestrained bottlenose dolphins use their learned whistles in matching interactions, in which an individual responds to a whistle of a conspecific by emitting the same whistle type. Vocal matching occurred over distances of up to 580 meters and is indicative of animals addressing each other individually.

Bottlenose dolphins show many cognitive and communicative skills that are rare among animals. They are capable not only of generalizing rules, developing abstract concepts and syntactic understanding in an artificial communication system (1), but also of vocal learning, i.e., the ability to modify the structure of a vocal signal as a result of experience with those of other individuals (2). Although extensive studies in nonhuman primates have not been able to present convincing evidence for vocal learning, this prerequisite for the evolution of spoken language has been demonstrated with much less research effort in bottlenose dolphins (2). Dolphins are capable of imitating new sounds accurately at their first attempt, and they keep this ability throughout their life (3). Vocal learning is also an important factor in the ontogeny of an individually distinctive signature whistle that each individual develops in the first few months of its life (4). Studies on captive individuals have shown that signature whistles are primarily used if animals are out of sight of each other, and they are therefore thought to function in group cohesion and individual recognition (5–7). However, because bottlenose dolphins are capable of vocal learning, individual signature whistles can

be found in the repertoire of more than one individual in captive dolphins (6, 8).

I investigated whether such shared whistles occur in matching whistle interactions between wild dolphins, a phenomenon indicative of their use in addressing specific individuals. Matching interactions were defined as an occurrence in which two whistles of the same type produced by separate individuals occurred within 3 s of each other.

There is often a clear effect of observer presence on dolphin behavior when methods such as tagging or boat pursuits are applied (6, 9). I used a noninvasive passive acoustic localization technique (10) to locate calling bottlenose dolphins (11). This method uses the differences in the time of arrival of the same sound at different widely spaced hydrophones. Signals from different recording channels were cross-correlated to determine the difference in the time of arrival of a sound at the two corresponding hydrophones. The time-of-arrival comparisons of three pairs of hydrophones then result in three hyperbolas of possible sound source locations. These hyperbolas intersect at the true location of the whistling dolphin. This analysis was conducted with SIGNAL software (Engineering Design, Belmont, Massachusetts). Recordings were conducted in the Kessock Channel of the Moray Firth, Scotland. All data were acquired from the shore, so that no boats or humans were present around the animals.

Vocal interactions between individuals were identified by comparing the distance of

the source locations of two successive whistles (minus twice the maximum localization error of 13 m) with the distance that a bottlenose dolphin could travel at its maximum reported swimming speed of 7.5 m/s (12) in the interwhistle interval. If the distance between two whistle sources could not have been covered by one individual in the time interval between those whistles, they must have been produced by different individuals.

Five naïve human observers were used to rate the similarity of each whistle interaction using only the extracted contours (13) of the whistles; this method is more reliable than computer-based methods that have been used in dolphin whistle studies (14). They were allowed to rate whistle similarity on a scale from 1 (=dissimilar) to 5 (=similar). The scores of the different observers were significantly similar (Kappa = 0.34,  $z = 16.9$ ,  $P < 0.00001$ ). Only whistle pairs that reached an average score of more than 3.0 were considered to be matching interactions (15).

In a total recording time of 258 min and 43 s from seven different days in July and August 1994 and 1995, a total of 1719 whistles was recorded. These recordings were made with an average of 10 animals present in the channel (quartiles: 7, 10, and 15). Independent counts conducted by a second observer from a higher observation point using binoculars showed that these counts were highly accurate. I could not identify individuals in this study, but a photo-identification study showed that at least 14 different individuals were using this area on a regular basis and that occasionally groups of more than 20 animals were present (16). Nine hundred ninety-one of the recorded whistles had a sufficient signal-to-noise ratio on all hydrophones for their source location to be determined. In this sample, 176 whistle interactions were found, of which 39 were classified as matching interactions (Fig. 1). In both matching and nonmatching interactions, 80% of the interwhistle interval was less than 1 s. The mean distance between matching individuals was 179 m (standard error: 22.8 m); the maximum was 579 m. Distances between animals in matching interactions were significantly smaller than those of animals in nonmatching interactions (Kolmogorov-Smirnov Two-Sample Test, two-tailed,  $D = 0.291$ ,  $P < 0.025$ ) (Fig. 2). A randomization test (17) showed that this number of matching interactions was significant.

School of Biology, University of St. Andrews, Bute Building, Fife KY16 9TS, UK, and Lighthouse Field Station, Aberdeen University, Cromarty, Ross-shire IV11 8YJ, UK. Present address: Woods Hole Oceanographic Institution, Biology Department, Woods Hole, MA 02543, USA.